The twenty-four hour variation of 5-hydroxytryptamine in the rat brain.

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THE TWENTY-FOUR HOUR VARIATION OF 5-HYDROXYTRYPTAMINE IN THE RAT BRAIN

submitted by J.G. HILLIER, B.Sc.

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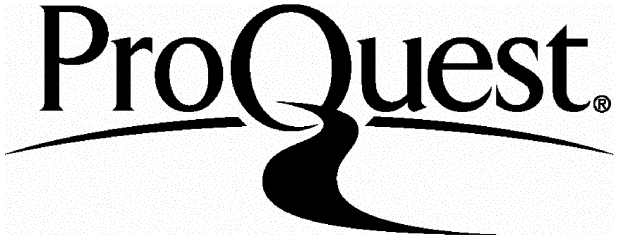

"Unus Dies par omni est."

Lucius Annaeus Seneca
(4 BC - 65 AD)

Epistulae Murales ad Lucilium
(Epis. XII sec. 7)

C O N T E N T S

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Chapter Three cont'd

A C K N O W L E D G E M E N T S

I wish to thank Dr. P.H. Redfern for the invaluable help and encouragement that he has provided throughout this project.

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S U M M A R Y

1. The concentrations of 5-hydroxytryptamine in the rat brain have been shown to vary with clock hour. Highest concentrations were observed at 13.00 h. and lowest concentrations at 01.00 h.

2. The twenty-four hour variation in brain tryptophan concentrations, tryptophan uptake, 5-hydroxytryptophan concentrations and uptake, 5-hydroxyindole-3-acetic acid concentrations and the activities of tryptophan-5-hydroxylase, 5-hydroxytryptophan decarboxylase and monoamine oxidase have been determined. The effects of inhibitors of catecholamine synthesis on the twenty-four hour rhythm of 5-hydroxy-tryptamine concentrations, and the effects of drugs which alter brain 5-hydroxytryptamine concentrations on locomotor activity were determined.

3. Brain and serum (free and bound) tryptophan concentrations vary with clock hour, but the rhythm is 180 degrees out of phase with that of 5-hydroxytryptamine. The uptake of ^{14}C-tryptophan into synaptosomes also varies with clock hour.

4. The rate of conversion of 5-hydroxytryptophan to 5-hydroxytryptamine was greater during the hours of light than during the hours of darkness, although the activity of the enzyme did not vary and substrate and pyridoxal phosphate availability were not limiting factors.

5. 5-hydroxyindole-3-acetic acid concentrations exhibit a twenty-four hour rhythm which follows the rhythm of 5-hydroxytryptamine concentrations.

6. Monoamine oxidase inhibitors, α-methyl-p-tyrosine and 6-hydroxydopamine abolish the twenty-four hour rhythm of 5-hydroxytryptamine concentrations and elevate brain levels of the amine.

7. It is proposed that the twenty-four hour variation of 5-hydroxytryptamine concentrations in the rat brain are controlled, at least in part, by the twenty-four hour variation of 5-hydroxytryptophan decarboxylase activity, which in turn is significantly influenced by the concentration of catecholamines in the brain.

CHAPTER ONE

INTRODUCTION

5-hydroxytryptamine is widely distributed in the central nervous system (CNS) of mammals, where it probably functions as a neurotransmitter. This indoleamine has been postulated to be involved in many and varied functions e.g. sleep, pain perception, and is noted for displaying a marked circadian rhythm in the CNS.

It is hoped that the results presented in this thesis will shed some light upon biochemical aspects of the control of the twenty-four hour variation of 5-hydroxytryptamine concentrations in the rat brain, and will perhaps help to integrate this apparently functionally distinct unit into the recognised patterns of brain function as we now know them.

It is the purpose of this introduction to give a resume of the work involved with the discovery, distribution, metabolism, regulation and function of 5-hydroxytryptamine in the CNS and to explain those characteristics of circadian rhythms which are of particular relevance to the amine.

1.1 Discovery, Isolation and Characterisation of 5-hydroxytryptamine

For more than a century mammalian physiologists have been aware of a vasoconstrictor substance which appears in the serum after blood has been allowed to clot. This substance, which they termed vasotonin, could be inactivated by first passing the blood through the lungs.

It was not until 1948 that this compound was isolated. Rapport, Green and Page, working at the Cleveland Clinic in America, crystallised a complex with vasoconstrictor activity, and gave it the name serotonin, a term still very much in vogue. The following year Rapport (1949) characterised the active moiety as 5-hydroxytryptamine. When this compound was prepared synthetically by Hamlin and Fischer in 1951, it was found to behave exactly as the naturally occurring "serotonin".

At approximately the same time Erspamer and his co-workers in Italy were investigating the substance that imparts peculiar histochemical properties to the enterochromaffin cells of the gastro-intestinal mucosa. They found a basic compound, which they called enteramine, in the gastric mucosa, and subsequently in other tissues. This compound had the ability to stimulate the gastro-intestinal tract. By 1946 Erspamer had suggested that the active component was an indolealkylamine,but was not able to make further progress until 5-hydroxytryptamine had been discovered in the blood. Erspamer and Asero (1952) then established that this was the chemical nature of Erspamer's "enteramine".

1.2 The Occurrence of 5-hydroxytryptamine

5-hydroxytryptamine occurs abundantly in animals including mammals, birds, reptiles, amphibians, fish, tunicates, molluses, arthropods and coelenterates, and in fruits such as pineapple, banana and plum and in various nuts. Man has probably been made most aware of the presence of 5-hydroxytryptamine by its occurrence in stings and venoms e.g. common stinging nettle, cowhage, wasps and scorpions.

1.3 5-hydroxytryptamine in the Central Nervous System (CNS)

Many earlier workers were unable to establish the presence of 5-hydroxytryptamine in the CNS, and therefore disputed its existence. Eventually Amin, Crawford and Gaddum (1954) assayed appreciable amounts of the amine in the brains of dogs.

Since these workers had demonstrated the existence of 5-hydroxytryptamine in the brain, it was essential to man's endeavour to propose a role for the amine. Not unnaturally, considering its situation, it was proposed to be a neurotransmitter, but this proposition necessitated the localisation of the amine into a specific neuronal network. Evidence has accumulated to support the belief that 5-hydroxytryptamine found in the brain is derived from specific neurones. This evidence may be summarised:

1) Information from the Falck-Hillarp technique of fluorescence histochemistry applied to the brain (Fuxe 1965).
2) Subcellular fractionation studies showing 5-hydroxytryptamine to be associated with nerve ending particles (Zieher and De Robertis 1963).
3) The loss of brain 5-hydroxytryptamine related to the degeneration of nerve fibres following lateral hypothalamic lesions (Heller and Moore 1968).

Of these findings, perhaps the use of the fluorescent histochemical technique has achieved most in associating the amine with specific neurones. Barter and Pearse (Pearse 1960) originally developed a fluorescent β-carboline complex (6-hydroxy-3,4-dihydroxy-β-carboline) from the addition of one molecule of formaldehyde to the 5-hydroxytryptamine molecule. The method was adapted for the mapping of neurones containing 5-hydroxytryptamine in the brain by the Swedish group of workers (for summary see Fuxe, Hokfelt

and Ungerstedt 1968). It is pertinent to note that the fluorescence produced from this method is very weak for this amine, particularly in the small nerve terminals. The majority of studies have therefore been conducted with the use of monoamine oxidase inhibitors and reserpine, or from large scale ablations of the forebrain to enhance fluorescence in the cell bodies. This is clearly not a sound basis for drawing conclusions about physiological mechanisms. However, Bedard, Carlsson and Lindqvist (1972) and Delorme, Froment and Jouvet (1966) studied the loss of brain 5-hydroxytryptamine following lesions placed according to data obtained from fluorescent techniques, and the results were compatible with the neuronal networks proposed.

More recent methods to increase the intensity of fluorescence include:-

1) Preloading with high doses of the amino acid precursor tryptophan (Aghajanian and Asher 1971), thus increasing the concentration of 5-hydroxytryptamine in the neurones, and consequently increasing the fluorescence, and

2) incubating slices of brain with 6-hydroxytryptamine and developing the fluorescent product, which is more intense than that of the natural amine. Unfortunately 6-hydroxytryptamine may be taken up into catecholaminergic neurones, and is therefore non specific. However, pre-incubation of brain slices with 6-hydroxydopamine selectively destroys catecholaminergic neurones, and subsequent perfusion with 6-hydroxytryptamine produces an increased fluorescence which is specific to 5-hydroxytryptamine neurones, although smaller nerve endings may still remain invisible (Farnebo 1971).

5-hydroxytryptamine has therefore been identified in the brain of mammals by methods which lack absolute definition, and therefore cannot provide final proof that the amine is a neurotransmitter, although it satisfies most of the criteria which are normally considered essential (Van Praag 1970).

1) 5-hydroxytryptamine shows a distinct predilection for the terminal ramifications of the axons, where it is located in synaptic vesicles.
2) The enzymes required for synthesis and degradation of the amine are present in the brain.
3) The turnover rate of the amine in nerve terminals is high.
4) 5-hydroxytryptamine introduced to functioning neurones by micro-electrophoresis influences their electrical activity.
5) Stimulation of nerves containing 5-hydroxytryptamine reduces the concentration of the amine in the terminal axonal ramifications, especially when re-synthesis is inhibited.

There are two very important omissions from this list. First, there is a lack of evidence to show that the micro-iontophoretic application of 5-hydroxytryptamine to post-synaptic structures, anywhere in the CNS, corresponds to the effect of stimulation of 5-hydroxytryptamine containing neurones. Secondly, if the amine is a neurotransmitter it must be available for release onto extraneuronal structures, from where it can be collected and identified. There is no evidence that this has been achieved.

Micro-iontophoretic application of 5-hydroxytryptamine to pre-synaptic structures produces responses which vary according to brain region. Most telencephalic neurones are depressed, while spinal and ponto-medullary neurones are excited (Bloom, Hoffer, Nelson, Sheu and Siggins 1973).

1.4 Distribution of 5-hydroxytryptamine in the CNS

The nerve cell bodies of the 5-hydroxytryptamine containing neurones are situated in the raphe nuclei in the brain stem and mesencephalon, with others in the reticular formation. Ascending and descending axons project to the more rostral parts of the brain (Fig. 1) and to the spinal cord, with nerve terminals apparently present in most regions of the CNS.

Descending bulbo-spinal neurones have their origin mainly in the raphe nuclei of the medulla oblongata i.e. nucleus raphe pallidus (B1), nucleus raphe obscurus (B2) and the cells around the pyramidal tracts (B3), from whence they descend in the white matter of the spinal cord (anterior and lateral funicles) giving off projections into the grey matter, probably partly as collaterals, at different levels. The greatest density of 5-hydroxytryptamine containing nerve terminals occurs in the lumbar and sacral regions.

The B3 group of cells lateral and dorsal to the pyramidal tract and trapezoid body, and the B5 group in the nucleus raphe pontis of the lower brain stem, provide neuronal innervation of the cerebellum and lower brain stem by short neurones.

The mesencephalic cell groups B7 (nucleus raphe dorsalis), B8 (nucleus raphe medianus) and the B9 region, provide the neurones for the ascending pathways to the diencephalon and telencephalon. The majority of 5-hydroxytryptamine-containing fibres ascend alongside noradrenergic neurones in the septum, and end in the cingulum and cerebral cortex in fine nerve terminals. The rostral areas of the brain are reached by this system, thus innervating the limbic forebrain, hypothalamus and neo-cortex. The concentration of 5-hydroxytryptamine in brain regions, measured biochemically, correlates well with the number of nerve terminals detected by the fluorescent histochemical technique. Particularly high concentrations are found in the nucleus suprachiasmaticus and septal regions and low concentrations are found in the cerebellum.

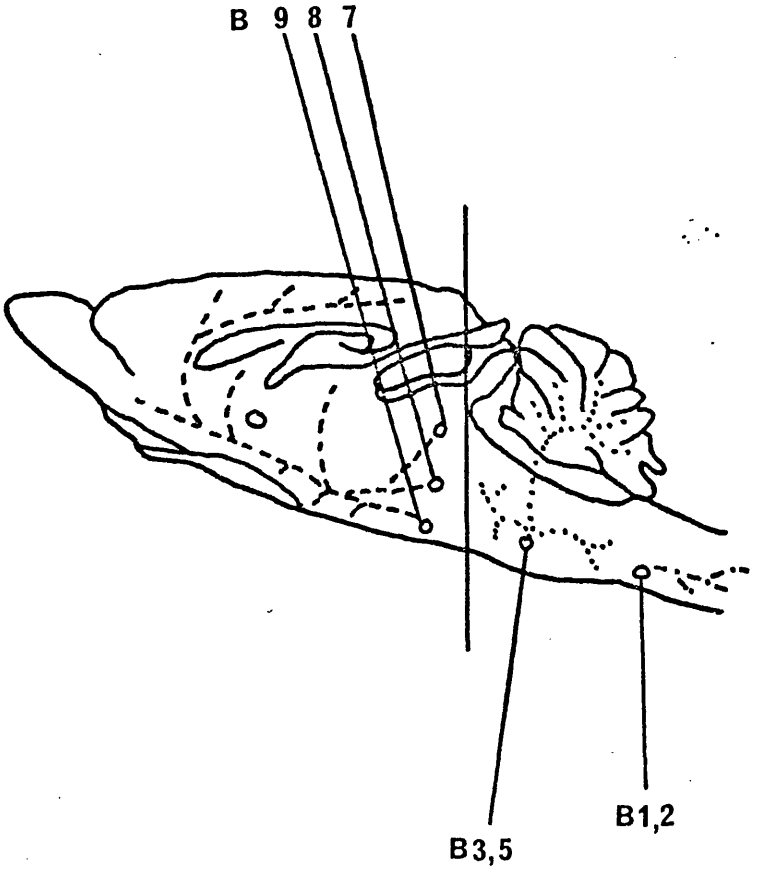


Figure 1. The network of 5-hydroxytryptamine-containing neurones in the rat brain (after Fuxe, Hokfelt and Ungerstedt 1971), ascending pathways to the prosencephalon originating from mesencephalic cell bodies.(– – – – –); descending pathways from cell bodies in the medulla oblongata. (–· –· –); 5-hydroxytryptamine neurones innervating the lower brain stem. (·····).

The ultrastructure of the nerve terminal is similar to that of catecholaminergic neurones. Both large and small dense-core vesicles are present and the synapse is of the Type 1 variety, at least in the hypothalamus. (Aghajanian, Bloom and Sheard 1969). Amine storage granules appear to be of more than one type (Thierry, Fekete and Glowinski 1968; Shields and Eccleston 1972), and may exist in two forms:

1) "Young" granules storing endogenous amine, which have undergone proximo-distal transport, possibly by the micro tubule system (Dahlstrom 1970) and have recently arrived at the nerve terminal.
2) Older granules resident at the nerve terminal.

The former may be important for the release and re-uptake of transmitter, while the main purpose of the latter is storage of the monoamine.

1.5 The Metabolism of 5-hydroxytryptamine

It has been classically established that 5-hydroxytryptamine is synthesised from the amino acid tryptophan, by hydroxylation to 5-hydroxytryptophan and decarboxylation to the amine. It is catabolised by monoamine oxidase to 5-hydroxyindole acetaldehyde and dehydrogenation to the major excretory product 5-hydroxyindole-3-acetic acid (Udenfriend 1959) (Fig.2.). All the necessary components of the synthesis and breakdown of 5-hydroxytryptamine are located in, or are associated with nerve endings, axons and cell bodies which have been shown to contain the amine.

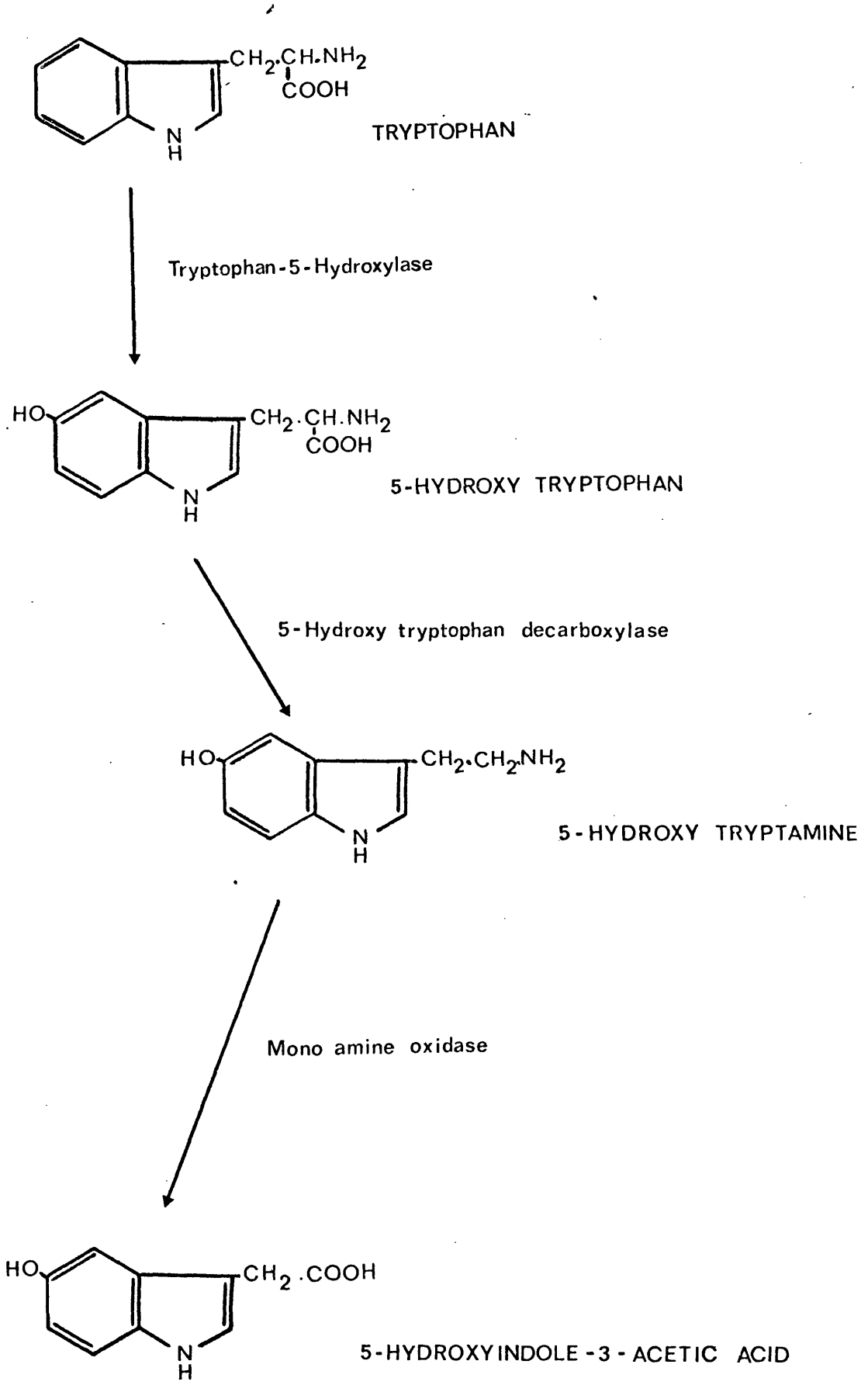


Figure 2 The major route of metabolism of 5-hydroxytryptamine.

This pathway, which constitutes the major metabolic pathway of 5-hydroxytryptamine will not be enlarged upon in this introduction, since investigation into the components of this pathway make up a large proportion of the work presented in this thesis, and will be discussed in much greater detail in Chapters 4-9. This introduction will be concerned with alternative metabolic processes which may affect the concentration of 5-hydroxytryptamine in the brain.

1.6 Alternative Routes of Metabolism

Some alternative routes of metabolism are shown in Figure 3.

The metabolism of 5-hydroxytryptamine relies on the availability of its dietary amino acid precursor tryptophan. Since the synthesis of the amine is only a minor pathway of tryptophan metabolism, it is apparent that some degree of control may be effected by the alternative pathways. It is conceivable that the removal of tryptophan for other purposes e.g. protein biosynthesis, excretion, could result in a decreased production of 5-hydroxytryptamine.

Peripherally, the major route of tryptophan metabolism starts with its oxidation, by liver tryptophan pyrrolase, to formylkynurenine, and by formamidation to kynurenine - the principal metabolite. Liver tryptophan pyrrolase is substrate inducible and is controlled, at least in part, by circulating corticosteroids (Curzon and Green 1971), and can thus regulate the concentrations of tryptophan in the circulation. There is no evidence to suggest the presence of the pyrrolase in the CNS, and none to suggest the existence of kynurenine. However, since the CNS relies on the circulation for its supply of tryptophan, the activity of liver pyrrolase may participate in the control of the amino acid levels in the CNS.

KEY TO ENZYMIC PATHWAYS FOR THE METABOLISM OF INDOLEAMINES IN THE BRAIN

1. Tryptophan-5-hydroxylase.
2. 5-hydroxytryptophan decarboxylase.
3. Monoamine oxidase.
4. Aldehyde dehydrogenase.
5. Tryptophan pyrrolase.
6. Formamidase.
7. Decarboxylase.
8. N-methylation
9. N, N-dimethylation.
10. 5-hydroxytryptophan pyrrolase and formamidase.
11. Cerebral aromatic amino acid aminotransferase.
12. Acetylases.
13. N-acetylases.
14. Hydroxy indole-O-methyl transferase.
15. Alcohol dehydrogenase.
16. Sulphotransferase.
17. Glucuronidase.
18. Decarboxylase.
19. 5-hydroxytryptamine sulphotransferase

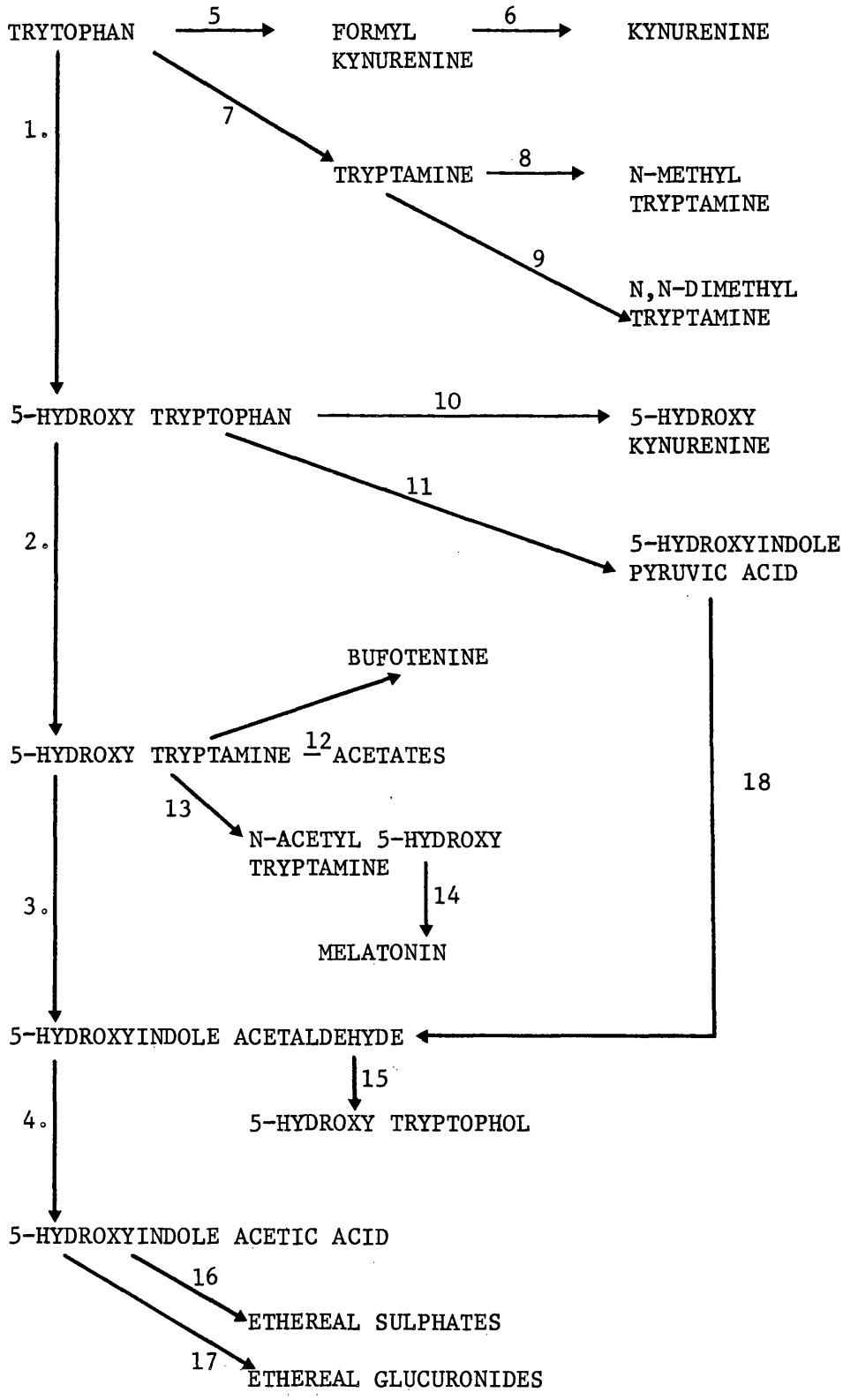


FIG. 3. The Metabolism of Indoleamines in the brain.

Tryptophan may be decarboxylated directly to tryptamine, and although this may occur readily in the periphery, very little tryptamine has been found in the CNS (Saavedra and Axelrod 1971). Perhaps more important is the discovery (Saavedra and Axelrod 1972) of an enzyme able to catalyse the conversion of tryptamine to N, N-dimethyltryptamine, and the detection of a compound resembling the tertiary amine (Heller, Fischer and Spatz 1973) in rat brain. N, N-dimethyltryptamine is a known psychotomimetic and has been postulated to be involved in the production of the symptoms of schizophrenia and in dream mechanisms (Heller, Fischer and Spatz 1973). This supposition would depend on the conversion of tryptophan to tryptamine in the CNS. This may be possible if the subsequent conversion of tryptamine to the methylated derivatives were so rapid as to make any increase in tryptamine undetectable, following loading with tryptophan. A second possibility is the conversion of tryptophan to N, N-dimethyltryptophan and decarboxylation to N, N-dimethyltryptamine. These pathways may be feasible,and perhaps important, if monoamine oxidase activity or the activity of endogenous inhibitors is altered.

5-hydroxytryptophan may be decarboxylated directly and rapidly to 5-hydroxytryptamine, or transaminated to the pharmacologically interesting 5-hydroxyindole pyruvic acid. The aminotransferase system is located in the mitochondria of neurones in the rat brain (Miller and Litwack 1969). Interest in the pathway has developed due to its ability to bypass the production of 5-hydroxytryptamine and monoamine oxidase activity, and provide an alternative source of 5-hydroxyindole acetaldehyde, which has been suggested to be functionally active in the CNS (Sabelli, Giardina, Alivisatos, Seth and Ungar 1969). ^{14}C-5-hydroxyindole pyruvic acid has been detected in the CNS following the intravenous injection of ^{14}C-5-hydroxytryptophan (Millard and Gal 1971). The transamination was

inhibited by increased cerebral concentrations of 5-hydroxytryptamine and by IDPN (β, β' iminodipropionitrile succinate). The aminotransferase system is freely reversible, thus providing a regulatory site for the synthesis of 5-hydroxytryptamine and 5-hydroxyindole acetaldehyde.

The aldehyde may be oxidised to 5-hydroxyindole-3-acetic acid, or reduced to form the alcoholic metabolite 5-hydroxytryptophol, which may also play an active role in the brain (Alivisatos 1973). The reduction is readily reversible. Small quantities of 5-hydroxytryptophol appear in the urine of man, but it is usually conjugated with sulphate or glucuronide.

Small amounts of 5-hydroxytryptamine may be excreted in the urine, but several other routes are available for the inactivation of the amine, apart from the major route to 5-hydroxindole-3-acetic acid. Initial acetylation yields an excretable metabolite, but particularly in the mammalian pineal gland, acetylation followed by O-methylation by hydroxy-o-methyl transferase produces the biologically active melatonin. Both the activity of the enzyme and the concentration of its product show a marked circadian variation. The latter depends on the former which is itself controlled by sympathetic innervation (Snyder, Axelrod and Zweig 1967). The role of melatonin, and the importance of its localisation in the pineal gland of mammals, is rather obscure. A general inhibitory effect on the gonads decelerates sexual maturation in females, and reduces the weight of seminal vesicles in male rats (Wurtman and Axelrod 1968). Melatonin has no effect on the melanocytes of mammalian skin, but has the ability to lighten the skin of amphibians.

N, N-dimethylation of 5-hydroxytryptamine produces the psychoactive compound bufotenine (5-hydroxy-N, N-dimethyltryptamine), a hallucinogen, which is naturally occurring in some mushrooms, seeds of Piptodenia

peregrina, and the skin of some toads. Since the enzymes which could convert 5-hydroxytryptamine to bufotenine are present in the CNS, it has been suggested that this compound may play a role in the production of the symptoms of schizophrenia.

It is possible that a hallucinogen may also result from the abnormal degradation of melatonin. Normally melatonin is hydroxylated in the liver on the sixth C atom, and excreted as the conjugated sulphate or glucuronide. McIsaac (1961) proposed that under certain unspecified conditions, melatonin may be converted to 10-methoxyharmalan, a hallucinogenic alkaloid, by the simple loss of a molecule of water and the closing of the side chain to form a tricyclic alkaloid.

A further route of inactivation of 5-hydroxytryptamine is by conjugation with sulphates. A sulphotransferase with this ability has been found in the brain (Gal 1972). Although the sulphotransferase normally plays a minor part in the inactivation of the amine, this system may be important following monoamine oxidase inhibition.

1.7 The Regulation of 5-hydroxytryptamine Concentrations in the CNS

It is apparent from the foregoing survey, and it will become more apparent in subsequent chapters, that many factors may be expected to affect the concentrations of 5-hydroxytryptamine in the CNS. It is equally clear that this concentration is rigidly controlled at any given time of day, but varies equally consistently throughout the twenty four hours of that day. Several workers have concluded that the regulation of the amine concentration is a function of the activity of tryptophan-5-hydroxylase (Green and Sawyer 1966). However, the enzyme is not saturated with its substrate (Jequier, Robinson, Lovenberg and Sjoerdsma 1969), and Grahame-Smith (1973) doubted the validity of the

widely held belief that this enzyme is the rate limiting step:-

"I find it very difficult to conceive that the regulation of a crucial functional pool of neuronal 5-hydroxytryptamine can depend upon the fine regulation of tryptophan hydroxylation, when this enzymic activity is so dependent upon the brain tryptophan concentration, which is not finely controlled. --------- compartmentation with regard to storage in granular binding sites and elsewhere, and a controlled intraneuronal metabolism of synthesised, in excess of needs, amounts of 5-hydroxytryptamine seems more likely mechanisms regulating the size and activity of those pools of 5-hydroxytryptamine which are of such crucial functional importance."

Renson (1973) found it difficult to believe that the concentration of tryptophan was the single controlling factor, and Aghajanian (1973) found no increase in tryptophan levels following an increased 5-hydroxytryptamine turnover after stimulation of the raphe nuclei. However Glowinski, Hamon and Hery (1972) suggested that the 'available' concentration of tryptophan was important, that this may be regulated by cyclic adenosine 3',5'monophosphate (AMP), and in addition, that the intraneuronal levels of the transmitter play a part in its own rate of synthesis. Schubert (1973) suggested that the size of the 5-hydroxytryptamine pools limits the synthesis of the transmitter.

A summary of some of the controlling factors of 5-hydroxytryptamine concentrations in the brain of the rat can be seen in Figure 4.

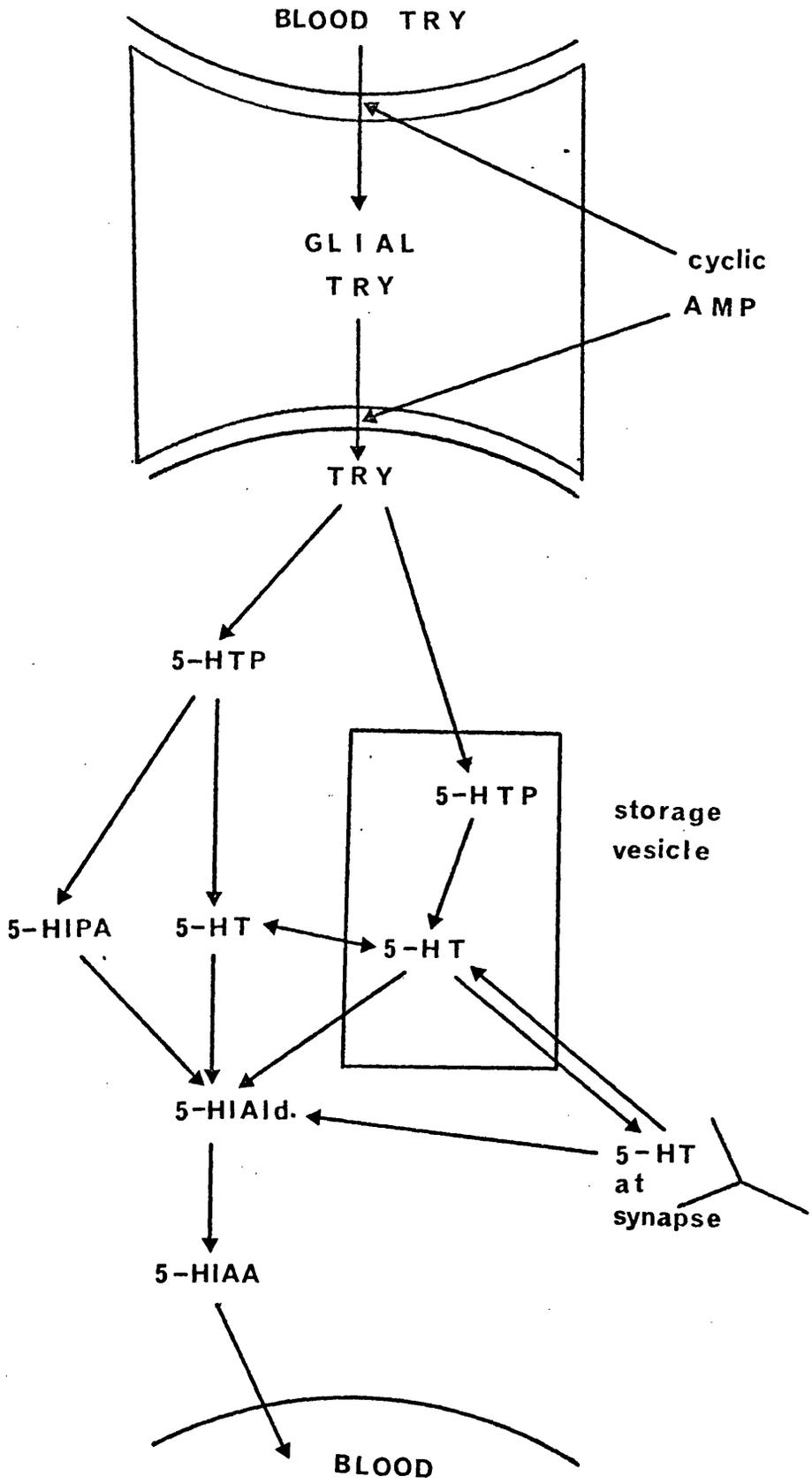


Figure 4. A model to suggest the possible regulating factors of 5-hydroxytryptamine synthesis in the rat brain.

1.8 Alterations of 5-hydroxytryptamine Metabolism by Drugs

A large number of drugs have been shown to alter the metabolism of 5-hydroxytryptamine in the CNS. A few of the more important of these will be considered.

The methyl xanthines, caffeine, theophylline and theobromine are probably the most widely consumed group of drugs, and have been shown to markedly influence the disposition of brain 5-hydroxytryptamine (Berkowitz and Spector 1971). These compounds produce increased levels of 5-hydroxytryptamine and 5-hydroxyindole-3-acetic acid in the brain, and have been used therapeutically alone, or in combination with other drugs, to control migraine headache, hyperthermia and to induce sedation and sleep. Abnormal 5-hydroxytryptamine metabolism has been implicated with all these maladies.

The benzodiazepines are a class of compounds widely preferred as minor tranquillisers (chlordiazepoxide, diazepam) and hypnotics (nitrazepam, flurazepam). Chase, Katz and Kopin (1970) recorded a decreased efflux of 5-hydroxyindole-3-acetic acid following administration of the drugs, while Dominic (1973) found a depression of the 5-hydroxytryptamine biosynthetic and metabolic pathway in the brain. These compounds do have a marked effect on the diencephalic and telencephalic regions, whilst having little affect on the brain stem. It has been concluded by some that behavioural suppression induced by anxiety may be mediated by 5-hydroxtryptamine, nevertheless the site of action of the benzodiazepines has not been elucidated.

Two groups of drugs are commonly used in the management of patients with psychotic depressions. These are the monoamine oxidase inhibitors eg. iproniazid, isocarboxazid, nialamide, phenylzine, tranylcypromine and the aminodibenzyl derivatives (tricyclic antidepressants), e.g. imipramine,

amitryptyline, desmethylimipramine. Most patients are helped by the administration of one of these drugs. The biochemical action of these compounds serves to increase the concentrations of noradrenaline and 5-hydroxytryptamine in the brain, either by inhibition of monoamine oxidase in the former group, or by inhibition of the aminergic reuptake mechanisms in the nerve terminal in the latter group. The behavioural stimulation of animals can occur not only by mechanisms involving noradrenaline, the concentrations of which are certainly increased in the brain, but also by the selective increase of 5-hydroxytryptamine concentrations (Bueno, Pscheidt and Himwich 1968). Indeed increased concentrations of 5-hydroxytryptamine, with normal noradrenaline concentrations, seem to assure behavioural stimulation. Coppen (1967) concluded that a dearth of 5-hydroxytryptamine may be involved in the mechanism of the endogenous depressions in some patients. Conversely an excess of 5-hydroxytryptamine has been implicated in manic episodes. This is evident from work with methysergide, which has been shown to reduce 5-hydroxytryptamine in the CNS (Oswald, Ashcroft, Beger, Eccleston, Evans and Thacore 1966), whilst relieving manic episodes (Verster 1963). The drug may even induce a subsequent depression (Serry 1969). Lithium, another drug which has been used in the treatment of manic episodes, has been shown to decrease 5-hydroxytryptamine turnover in rat brain. (Corrodi, Fuxe and Schou 1969).

1.9 The Role of 5-hydroxytryptamine in the CNS.

Many theories have suggested a role for 5-hydroxytryptamine in the CNS. Among those most popular are the involvement of the amine in maintaining a balance of the general behaviour of the animal, in pain recognition, in hypothermia, sleep, learning and memory, male sexual behaviour and stress, while a lack of the amine has been considered to cause symptoms of depression, schizophrenia and convulsive disorders including Parkinson's disease. The presence of the amine in the CNS is not essential to life, but to the quality of life, and perhaps to mood.

To suggest that 5-hydroxytryptamine, in isolation, has a specific role in the CNS is probably an oversimplification. It appears more likely that the amine is part of an interacting system, and it is the balance of the components of the system which produces the observed behavioural pattern. I intend to present only the data which is relevant to 5-hydroxytryptamine. The possible involvement of other systems will be borne in mind and will figure in the final discussion.

During the last few years Jouvet et al. and other workers have accumulated a large amount of evidence to suggest that 5-hydroxytryptamine is involved in, and may be responsible for slow wave sleep, and for the priming of paradoxical sleep. There seems little doubt that the axonal system emanating from the raphe nuclei is involved, to a large extent, in this basic physiological response.

Much of the evidence which supports a role for 5-hydroxytryptamine in determining an animal's sensitivity to painful stimuli has been obtained by placing lesions in the ascending axonal system from the raphe nuclei, and demonstrating a decrease in brain concentrations of 5-hydroxytryptamine (Lorens, Sorensen and Harvey 1970) and a

concomitant increased sensitivity to painful stimuli, especially foot shock (Evans 1961). Lesions in other regions of the brain do not produce either of these effects. It is important to note, at this point, that workers who have attempted to establish a biochemical correlate of learning and memory, often used a foot shock technique. Woolley and Van der Hoeven (1963) found that animals learn more quickly when 5-hydroxytryptamine has been depleted, but it is not possible to exclude the suggestion of an increased sensitivity to pain producing the observed response.

p-Chlorophenylalanine (p-CPA) is a specific depletor of brain 5-hydroxytryptamine, and manifests its action by inhibiting tryptophan-5-hydroxylase (Koe and Weissman 1966). Evidence that p-CPA acts as an aphrodisiac in males is somewhat equivocal, but the work of Gessa and Tagliamonte (1974) in rats and rabbits would support the hypothesis. Many workers have found an enhancement of mounting behaviour in male rats treated with p-CPA or p-CPA plus a monoamine oxidase inhibitor, although a decrease, increase or no change in ejaculation frequency has been recorded by different groups of workers. The suggestion remains that 5-hydroxytryptamine participates in the regulation of male sexual behaviour.

Feldberg and Myers (1964) proposed the monoamine theory of thermoregulation, whereby hypothalamic release of 5-hydroxytryptamine and noradrenaline in a balanced situation could regulate the temperature of a mammal around a fixed reference temperature of approximately 37^{o}C. 5-hydroxytryptamine evokes hyperthermia when injected into the anterior hypothalamic pre-optic area of monkeys and cats. In these animals, the amine may be a transmitter activating the central heat conservation and production pathways (Myers 1973).

An increase in alerting has been recorded following the stimulation of the cell bodies of 5-hydroxytryptamine containing neurones in the raphe nuclei (Sheard and Aghajanian 1968). Thierry, Fekete and Glowinski (1968) showed that stress activates the indoleamine system, whereas isolation depresses it. An increase in 5-hydroxytryptamine metabolism following stress may last for many days if the animals are maintained in an unfavourable environment, such as being intermittently shocked on a grid (Bliss 1973). 5-hydroxyindole-3-acetic acid concentrations in the brain remain increased several hours after the presentation of a single stressful stimulus.

Altered metabolism of 5-hydroxytryptamine in psychiatric and neurological disorders has been suspected or demonstrated. The amine has been implicated in phenylketonuria, mongolism, Hartnup's disease, migraine, Parkinson's disease, depression and schizophrenia to a greater or lesser degree.

1.10 Interactions of 5-hydroxytryptamine with Catecholamines and Cyclic-AMP in the CNS

Although this thesis deals mainly with 5-hydroxytryptamine and the indoleamine pathways in the brain, it is not possible to consider this system in isolation, but rather as a part of an integrated and highly complex unit in which actions of other systems may modify, or even control, the physiological manifestations of this particular system.

a) Catecholamines

Several physiological aspects of catecholamine and 5-hydroxytryptamine interaction can be readily recognised.

Jouvet (1969) has demonstrated a classical example of the way in which the two systems interact to produce the characteristic signs of normal sleep. 5-hydroxytryptamine induces slow wave sleep and possibly prepares for the noradrenaline-induced paradoxical sleep, which cannot be induced in the absence of slow wave sleep. Bliss (1973) has found an interaction between dopamine and 5-hydroxytryptamine which is manisfested in stress reactions. Both aminergic systems are activated during gross behavioural disturbances. While muscular activity and fear activate dopaminergic neurones "something related to gender" activates 5-hydroxytryptamine neurones. Both systems are required to produce the normal reactions to stress.

Of clinical importance is the suggested interaction between dopamine and 5-hydroxytryptamine systems in Parkinson's disease, following Hornykiewicz's (1966) finding that the concentration of both of the amines was decreased in the substantia nigra of patients suffering from the disease. The caudate nucleus is also implicated in Parkinson's disease, and dopamine has been suggested to be a neurotransmitter in this region where the balance of the two amines is crucial (Van der Wende and Johnson 1970). Dopamine is located in the soluble fraction of homogenates, and 5-hydroxytryptamine in the bound form (Laverty, Michaelson, Sharman and Whittaker, 1963). Van der Wende and Johnson (1970) have suggested that 5-hydroxytryptamine might be released to complex with the dopamine to modulate its action, and thus regulate catecholamine activity on a purely molecular level. This complex can occur

between all catecholamines and 5-hydroxytryptamine, and appears to inhibit the oxidation of the catecholamines. The diurnal variation of 5-hydroxytryptamine may, in part, be controlled by catecholamine neurones in the CNS (Hery, Rouer and Glowinski 1973), although Bobillier and Mouret (1971) found the rhythms of the two amines to be independent. The diurnal variation of 5-hydroxytryptamine in the mammalian pineal gland is controlled by a noradrenergic system, since sympathectomy abolishes the rhythm (Zweig and Axelrod 1969). Although the activity of the pineal gland cannot be related to brain activity, there does appear to be some evidence for an interaction between these amines in the brain.

Asano (1971) compared the maturation of the circadian rhythms of noradrenaline and 5-hydroxytryptamine in the rat. Animals up to 17 days post natal showed a similar pattern for both amines, with highest concentrations during the dark period. From day 21 until day 66 these rhythms reached maturity with maximum concentrations of 5-hydroxytryptamine in the light period, and highest concentrations of noradrenaline in the dark period. The maturation of these rhythms was closely associated with those of locomotor activity and the sleep/wake mechanism.

b) Cyclic 3',5' Adenosine Mono Phosphate (AMP)

Increased concentrations of cyclic AMP have been detected in pharmacological (Cramer, Ng and Chase 1972) and physiological situations (Hamadah, Holmes, Barker, Hartman and Parke 1972) which stimulate 5-hydroxytryptamine synthesis.

Tagliamonte et al (1971(a)) increased the rate of synthesis of 5-hydroxytryptamine, without altering the concentration of the amine in the brain, following cyclic AMP or dibutyryl cyclic AMP injection into the lateral ventricles. There was a marked increase in the concentration of the amino acid precursor tryptophan, and an increased uptake of labelled tryptophan into slices of brain. Since tryptophan-5-hydroxylase is probably not saturated with its substrate, the increase in synthesis of 5-hydroxytryptamine is probably secondary to the increased concentrations of tryptophan in the brain. Brain adenyl cyclase and phosphodiesterase, enzymes which catalyse the formation and degradation of cyclic AMP, are concentrated in the synaptosomal fraction of tissue homogenates (De Robertis, Rodriguez de Lores Arnaiz, Alberici, Butcher and Sutherland 1967). It is therefore conceivable that cyclic AMP is closely associated with 5-hydroxytryptamine-containing nerve terminals, and may play an integral part in the control of the synthesis of the amine in the CNS.

1.11 Circadian Rhythms

Biological rhythms have been detected throughout the plant and animal kingdoms, varying in frequency from many cycles per second e.g. heart beat, to one cycle in many years e.g. the flowering cycle of several large cacti. Biological rhythms have been recently well reviewed (Mills 1973), and further details will not be presented here.

When the frequency of a rhythm is approximately one cycle per day, the rhythm is termed circadian, and may be recognised by certain characteristics.

1) The rhythm has a frequency of approximately one day, and may be entrained to exactly twenty-four hours.

2) The oscillation often conforms to the mathematical shape of a sine curve.

3) The rhythm maintains its frequency independently of the rhythms for habit or environment.

4) If the rhythm is entrained to a new period, it will undergo a period of disruption before entrainment; similarly it will not immediately revert to the former period when it is re-presented.

5) Circadian rhythms are nearly always nycthemeral.

5-hydroxytryptamine displays a circadian rhythm. In this thesis I have investigated the rhythm of 5-hydroxytryptamine following its entrainment to twenty-four hours. Hence in the title and throughout this thesis, the term "twenty-four hour rhythm" has been used instead of the often incorrectly applied term " circadian rhythm".

The origins of circadian rhythms are not known, although several theories have been proposed, mainly concerning endogenous biochemical fluctuations (Molchanov 1971). Ehret and Trucco (1967) suggested the "Chronon Concept", whereby the rhythms are based on the recycling of a mechanism regulating the transcription of template RNA (ribonucleic acid) from DNA (deoxyribonucleic acid). Even on these bases it is not clear what is causing the rhythm, whether it relates to the synthesis of inhibitors and/or substrate, or perhaps to some form of compartmentation (Hastings 1970).

Many workers have envisaged the controlling centre of circadian variations as a biological clock. The location of this "clock" has nearly always been the brain, and more specifically the thalamus (Strughold 1965) or hypothalamus (Mills and Conroy 1970). This work has not attempted to locate a "clock", but has looked for a controlling influence in one or more factors in the metabolism of 5-hydroxytryptamine, or in an association with other brain constituents capable of regulating the circadian rhythm of the amine. This influence may itself subsequently be controlled by a "clock".

1.12 Statistical Analysis of Circadian Data

With the presentation of a series of data, which together apparently form a rhythm in a single cycle e.g. a twenty-four hour rhythm with a single acrophase, it is desirable to obtain an objective estimate of the phase and amplitude of the rhythm. It is not satisfactory simply to compare the highest values with the lowest values, since one may rightly be accused of "data snooping". The usual method is to attempt to fit the observed data to a sine curve, a geometric model with a natural, recognised rhythm. It is assumed that if the data do not significantly differ from the calculated sine curve, then a rhythm is in evidence, whose highest values do differ from lowest values. In this thesis only data collected at equal time intervals over a twenty-four hour period have been examined. The analysis is thus straightforward. (For analysing more complex irregularly collected data see Fort and Mills 1970).

In this instance, the best fit sine curve has been calculated using the Fourier analysis, and the experimental data compared to the calculated data by means of the X^2 test.

CHAPTER TWO

CONTROL OF THE ENVIRONMENT

2.1 INTRODUCTION

The normal environmental conditions in the laboratory or animal house vary, particularly with respect to lighting, noise and temperature, throughout the seasons. In any animal experiment it is necessary to control these parameters, and it is particularly vital if circadian variations are being estimated. In addition, there is ample evidence to suggest that the concentration and metabolism of 5-hydroxytryptamine in the brain is susceptible to environmental fluctuations (Hauty and Adams 1966a,b; Schwartz and Aghajanian 1969; Thierry, Fekete and Glowinski 1968). In planning the experiments presented in this thesis, it was therefore doubly important that environmental variability should be controlled.

The possibility that circadian changes in the performance of the operator may produce changes in circadian data being assessed, is a criticism which has often been levelled at workers in this field. In the cabinets described in this chapter, it is possible to arrange the lighting cycle, so that samples may be taken during the normal working period of the operator. In addition, noise levels, temperature and stress could be maintained within acceptable limits (Hillier, Davies and Redfern 1973).

2.2 Cabinet Construction

The dimensions of the cabinet, which was made from $^{6}/_{10}$ in. blockboard, are shown in Figure 5. To ensure light proofing, $\frac{1}{2}$ in. plastic foam strips were sandwiched between adjoining faces. To increase sound insulation, each cabinet was lined with $\frac{1}{2}$ in. polystyrene sheeting. The doors were hinged along 18 in. of the

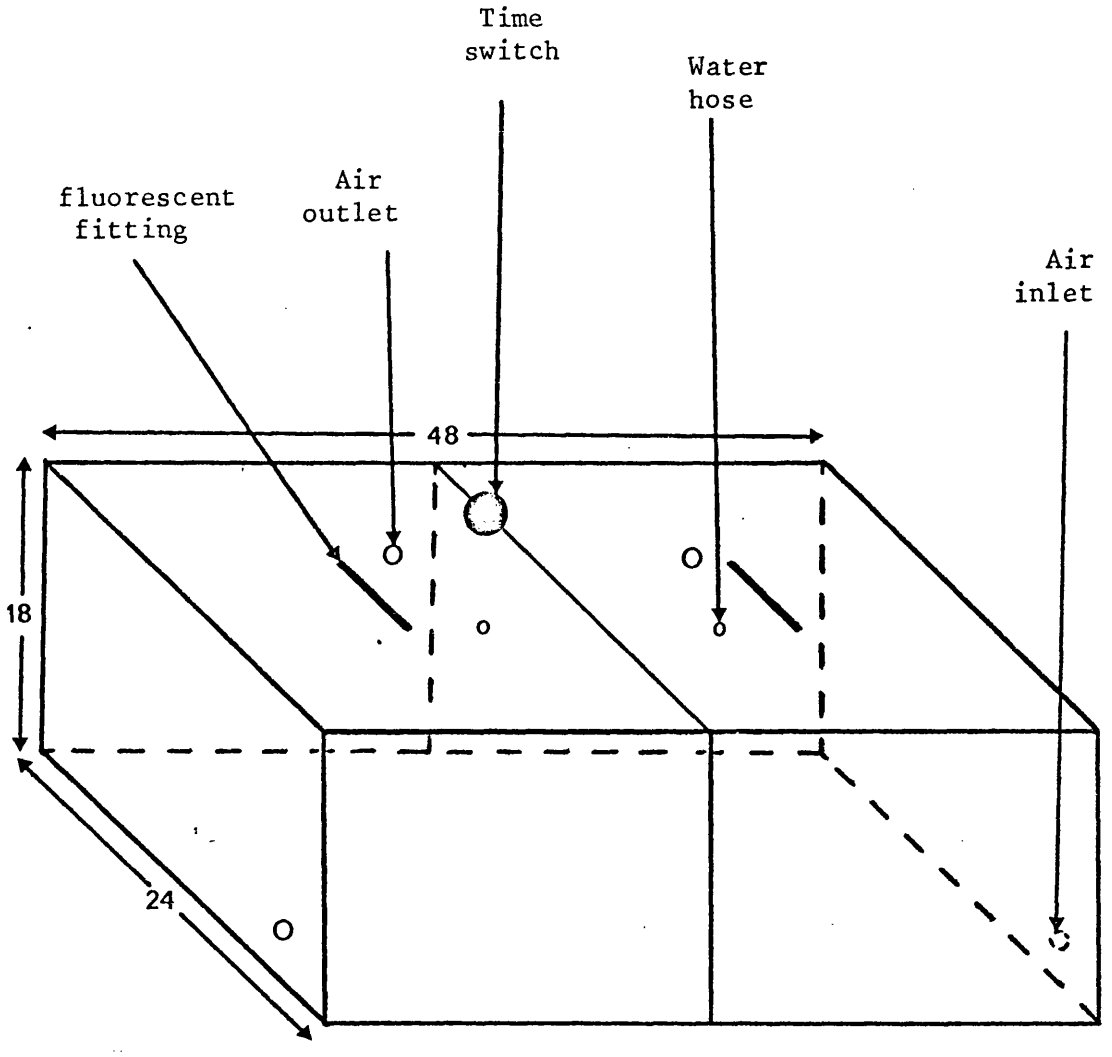


Figure 5. The Dimensions of the environmental cabinet.

bottom edge, and were closed with a latch fitting. The cabinets were lit by 12 in. miniature fluorescent fittings with 8 watt, warm white tubes (Thorn L.J. S1008.H). To prevent overheating, the choke was removed from each fitting and reassembled outside the cabinet. Each light was connected through a time switch (Sangamo Type 5254-1-171).

The maintenance of animals under these conditions of light and sound insulation, poses the problem of dispensing heat and water vapour. This problem was overcome by using domestic extractor fans (Xpelair FXC 6) to pull a constant stream of air through the cabinets. One fan, which was capable of removing 1,200 cu.ft. of air per hour, served two cabinets, to which it was connected through triple gauge 1¼ in. bore rubber tubing. The intake was first passed through an insulated chamber containing a 300 watt infra red bulb. This was activated by a thermostat inside the cabinet, so that in the event of a fall in temperature inside the cabinet, the temperature of the air input was automatically increased.

The complete apparatus is shown diagrammatically in Figure 6. A constant watering reservoir was included, and by using extra large food hoppers on the cages, animals (in groups of seven or eight) could be left undisturbed for up to fourteen days.

2.3 Cabinet Characterisation

The cabinets were shown to be light proof, by leaving a photographic plate inside the cabinet, locking the cabinet, and exposing the cabinets to the normal lighting of the laboratory. The photographic plate was subsequently removed and developed and was found to exhibit no signs of light entry into the cabinet.

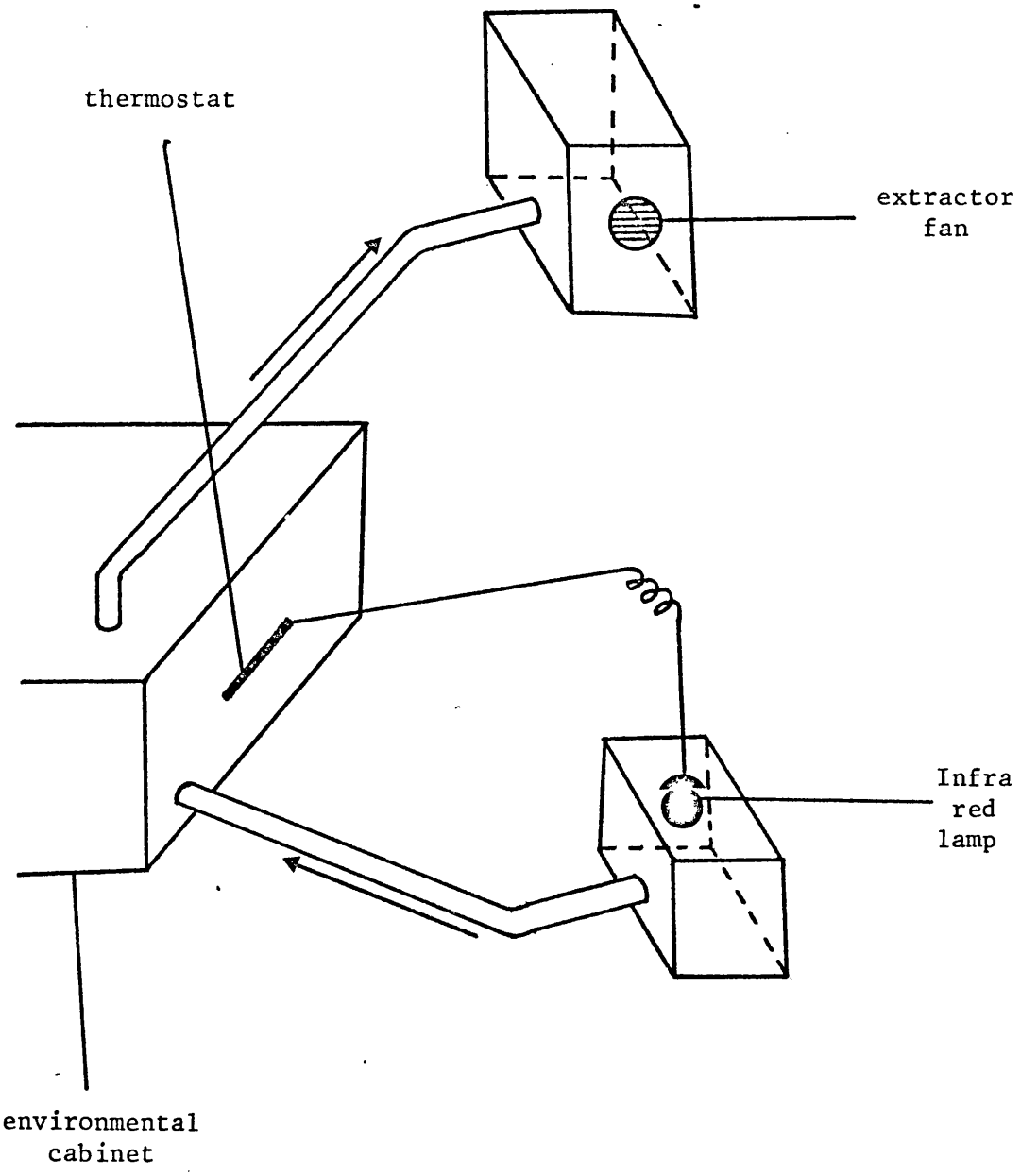


Figure 6. The Organisation of the controlled environmental system.

The cabinets were tested for sound proofing by exposing them to varying frequencies and intensities of sound at a distance of ten feet from the cabinets. A portable tape recorder was used to record sound within the cabinets. The cabinets were found to be sound proofed, under these conditions, below 74 dB, a level considered to be well within the normal noise levels of the laboratory, and therefore satisfactory for our purposes. The constant background noise provided by the extractor fans, provided an additional buffer to external auditory cues.

With the thermostat set at 24°C, the temperature inside the cabinets varied by ± 1.5°C over a seven day trial period. However, it must be remembered that since there is no means of lowering the temperature inside the cabinets, a large increase in temperature in the laboratory could cause a corresponding increase in temperature in the cabinets. In this respect therefore, the apparatus is not fully independent of the external environment. This could be overcome by the provision of some mechanism for cooling the air input, but the necessity for such a provision has not yet been encountered.

These cabinets, constructed and set up in groups of four, using the extraction and heating systems described, have been used with complete satisfaction throughout the period encompassed by this thesis.

2.4 Maintenance of Animals

The experiments were performed with the rats maintained as follows:

Male Sprague Dawley rats (120 - 140g) were kept in groups of seven or eight, with one group per cabinet. Using extra large food

hoppers and a constant watering reservoir system, the rats had access to food (Oxoid Breeding Diet) and water ad libitum, and could remain undisturbed for up to fourteen days.

In many experiments, the rats were on a reversed lighting regimen, and therefore had undergone a phase shift upon entry into the cabinets. At least ten days were therefore allowed for acclimatisation for all groups of animals (Davies, Navaratnam and Redfern 1974). There was no recognisable difference between groups of animals maintained on a 'normal' lighting regimen and those on the reversed schedule. Weight gain, weight of food consumed and volume of fluid intake were all similar.

In all experiments the rats were maintained either on a 06.00 - 18.00 light/dark schedule or a 06.00 - 18.00 dark/light schedule, thus giving twelve hours light and twelve hours dark per twenty-four hour day. The temperature was maintained at $24 \pm 1.5^{\circ}C$ throughout.

CHAPTER THREE

The twenty-four hour Rhythm of 5-hydroxytryptamine in the Rat Brain.

3.1 INTRODUCTION

A twenty-four hour or circadian fluctuation in the concentration of 5-hydroxytryptamine has been demonstrated in the whole brain of the rat. (Montanaro, Casoli and Babbini 1964; Dixit and Buckley 1967; Montanaro and Graziani 1967; Scheving, Harrison, Gordon and Pauly 1968; Schwartz and Aghajanian 1969; Okada 1971; Asano 1971; and Davies and Redfern 1970), in the whole brain of the mouse (Albrecht, Visscher, Bittner and Halberg 1956; and Morgan and Yndo 1973), the whole brain of the hamster (Matussek and Patshke 1963), various brain regions in the rat (Quay 1963, 1964, 1965, 1968; Fiske 1964; Snyder and Axelrod 1964; Snyder, Axelrod, Fischer and Wurtman 1964; Snyder, Axelrod and Zweig 1965; Snyder and Axelrod 1965; Snyder, Axelrod, Wurtman and Fischer 1965; Axelrod, Snyder, Moore and Heller 1966; Snyder and Zweig 1966; Snyder, Axelrod and Zweig 1967; Walker and Friedman 1967; Friedman and Walker 1968; Snyder 1968; Wurtman, Axelrod and Kelly 1968; Merritt and Sulkowski 1969; Bobillier and Mouret 1971; Scapagnini, Moberg, Van Loon, DeGroot and Gahong 1971; Hery, Rouer and Glowinski 1972) in brain regions of the cat (Reis, Weinbren and Corvelli 1968; Reis, Corvelli and Conners 1969) and in the turtle (Quay 1967).

In the work on whole rat brain, the highest concentration of 5-hydroxytryptamine was found during the light period, and the lowest concentration during the dark period.

In brain regions of the rat, circadian rhythms of 5-hydroxytryptamine concentrations have also been found, with highest concentrations during the light period and lowest concentrations during the dark period e.g. in the pineal gland (Quay 1963, 1964, 1968; Snyder and Axelrod 1964; Fiske 1964; Snyder, Axelrod, Fischer and Wurtman 1964; Snyder,

Zweig and Axelrod 1964; Snyder and Axelrod 1965; Snyder, Axelrod and Zweig 1965; Snyder, Zweig, Axelrod and Fischer 1965; Snyder, Axelrod, Wurtman and Fischer 1965; Snyder and Zweig 1966; Axelrod. Snyder, Moore and Heller 1966; Snyder, Axelrod and Zweig 1967; Snyder 1968; Wurtman, Axelrod and Kelly 1968; Merritt and Sulkowski 1969). In 1964 Quay reported that there was not a circadian rhythm of 5-hydroxytryptamine in the anterior or posterior hypothalamus, but later (1968) he reported a rhythm in whole hypothalamus, a finding which was confirmed by Hery, Rouer and Glowinski (1972). Quay (1965) found rhythms in three areas of the cortex, with higher concentrations in the dorsal frontopolar regions than in more posterior areas, while Hery, Rouer and Glowinski (1972) reported a rhythm in whole cortex. Other authors have shown that the circadian or twenty-four hour rhythm of 5-hydroxytryptamine exists in every brain region in which it has been investigated, e.g. in whole brainstem (Hery, Rouer and Glowinski 1972), lateral lower brainstem (Quay 1968), mid brain and caudate nucleus (Friedman and Walker 1967), amygdala and hippocampus (Scapagnini, Moberg, Van Loon and De Groot 1971).

It would thus appear that the summation of the circadian effects on the concentration of 5-hydroxytryptamine in the brain region is responsible for the large fluctuation measured in whole brain, and since the rhythm of whole brain 5-hydroxytryptamine concentrations is similar to that of every brain region investigated, the whole rat brain was mainly used as the tissue of study in this thesis.

Although a large number of workers have reported the circadian or twenty-four hour variation of 5-hydroxytryptamine concentrations, it was necessary to establish such a rhythm under the conditions employed in our own laboratories, and then to investigate the regulating factors of this rhythm under these same conditions.

3.2 METHODS

Male Sprague Dawley rats (120 - 140g) were housed, in groups of seven or eight under the constant environmental conditions described in Chapter Two, for at least ten days before each experiment. Different groups of rats were killed at intervals of four hours commencing at 09.00 h. When those groups housed in a reversed lighting cycle were killed, the laboratory was illuminated only by a red photographic safety lamp, since red light has been shown to have no significant effect on circadian variations (McGuire, Rand and Wurtman 1973).

The rats were killed by decapitation, the pineal gland removed, and the whole brain dissected out from a position immediately caudal to the cerebellum. The brains were frozen in liquid nitrogen and stored at $-14^{o}C$ until assayed. The time taken for the removal of the brain never exceeded two minutes.

3.2.1. Extraction

(Shore and Olin 1958). Brains were weighed and subsequently homogenised in washed acid n-butan-1-ol (10 ml/g tissue), using an M.S.E. high speed rotary cutter (Type 7700) at approximately 9,500 r.p.m. for three minutes. The homogenate was centrifuged at 4,000 r.p.m. for five minutes in a bench centrifuge. The whole of the organic layer was transferred to 125 ml'medical flats' containing 15 ml water and 30 ml n-heptane, and shaken for five minutes in a mechanical shaker (Gallenkamp super shaker SD-050), after which the bottles were centrifuged at 500g for three minutes.

3.2.2 Assay of 5-hydroxytryptamine

The assay used was a modification of that of Snyder, Axelrod and Zweig (1965).

2.0 ml of the aqueous phase was shaken for five minutes with 2.0 ml. borate buffer pH 10.0, 10 ml. washed n-butan-1-ol, and approximately 2g of sodium chloride in 125 ml 'medical flats' as described before.

The bottles were centrifuged as described previously, and 5 ml. of the organic phase was transferred to bottles containing 10 ml. n-heptane and 2.0 ml. phosphate buffer pH 7.0. The bottles were shaken for five minutes and centrifuged for three minutes as described before.

1.2 ml. of the aqueous phase was heated for thirty minutes at 70°C with 0.1 ml. 0.1 M ninhydrin reagent (indane-trione-hydrate) in screw top test tubes. After cooling for one hour at 4°C, the fluorescence was measured against aqueous standards, and internal and external reagent standards at activation wavelength 380 mμ. and emission wavelength 490 mμ. (wavelengths uncorrected) in an Aminco-Bowman spectrophotofluorometer.

3.2.3 Characterisation of 5-hydroxytryptamine

The extracted product was shown to have a fluorescence spectrum identical to that of pure 5-hydroxytryptamine (Sigma), and may therefore be assumed to be 5-hydroxytryptamine (Figure 7).

The effect of related indoleamines on the fluorescence of 5-hydroxytryptamine was also measured. Tryptophan, 5-hydroxytryptophan and 5-hydroxyindole-3-acetic acid were extracted, and the fluorescence developed and measured as described for 5-hydroxytryptamine.

None of these indoleamines produced a fluorescent product which gave a measurement exceeding that of a phosphate buffer blank. The results which follow can therefore be taken to be a true measure of 5-hydroxytryptamine content of the brain.

3.2.4 Statistical Methods

Statistical significance of the twenty-four hour variation of 5-hydroxytryptamine concentrations in the rat brain was determined by Fourier analysis and the X^2 test as described in Chapter One.

3.3 RESULTS

The twenty-four hour rhythm of brain concentrations of 5-hydroxytryptamine, obtained by the methods described, is shown in Figure 8, and is compared with the calculated sine curve in Figure 9. Brain 5-hydroxytryptamine concentrations are expressed in millimicrograms per gram of brain wet weight $\pm$ standard error of the mean (S.E.M.), and are plotted against clock hour. The twenty-four hour rhythm was not significantly different from the calculated sine curve. The highest concentration of 5-hydroxytryptamine (444.3 ± 8.1 mμg./g) was found in the middle of the light period at 13.00 h., and the lowest concentration (289.1 ± 11.0 mμg./g) in the middle of the dark period at 01.00 h. A forty per cent fluctuation was shown, and values at the nadir and zenith of the curve were significantly different at the $p < 0.001$ level by Students' t test analysis.

Characteristically the rate of change of 5-hydroxytryptamine concentrations slowed during both the ascending and descending phases of the rhythm at the time of "lights on" and "lights off"

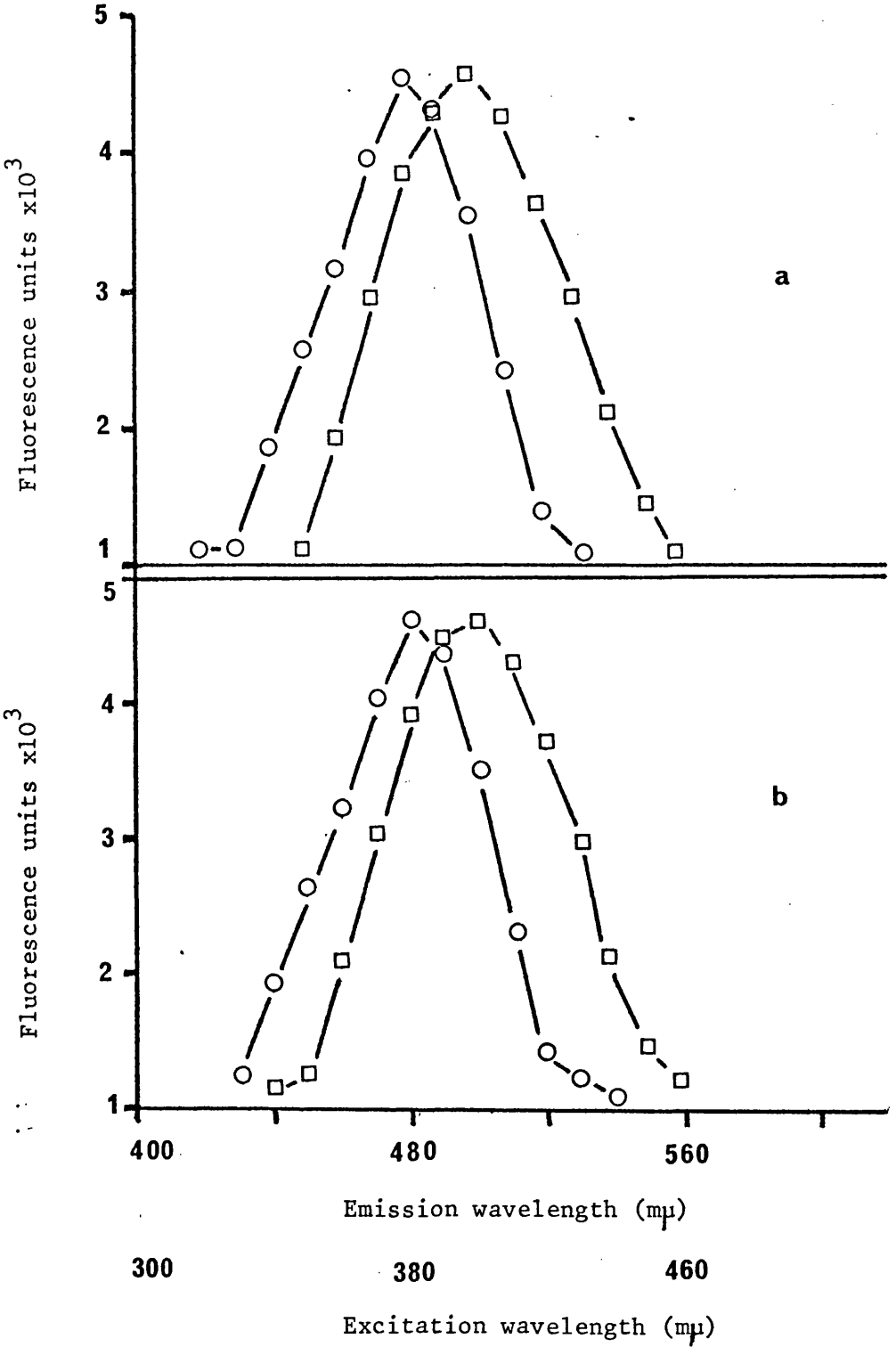


Figure 7. The fluorescence spectrum of extracted 5-hydroxytryptamine (a) compared with the pure product (b). (□——□) excitation 380mμ, scan emission; (○——○) emission 495mμ, scan excitation.

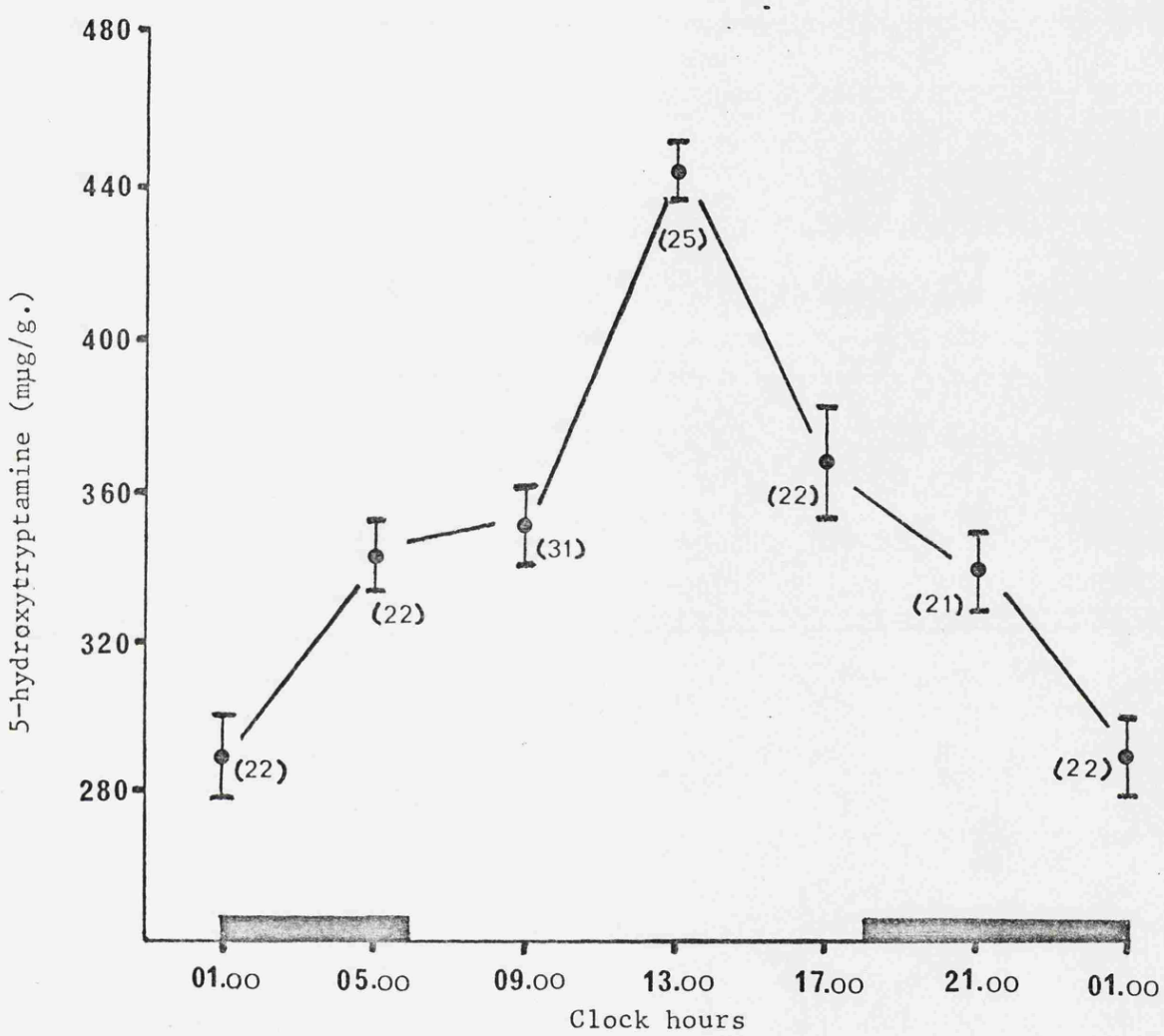


Figure 8 The twenty-four hour rhythm of 5-hydroxtryptamine concentrations in the rat brain. Concentrations are expressed in mμg/g brain wet weight. ± S.E.M. The numbers of animals used at each clock hour are shown in parentheses. The solid black bars indicate the period of darkness.

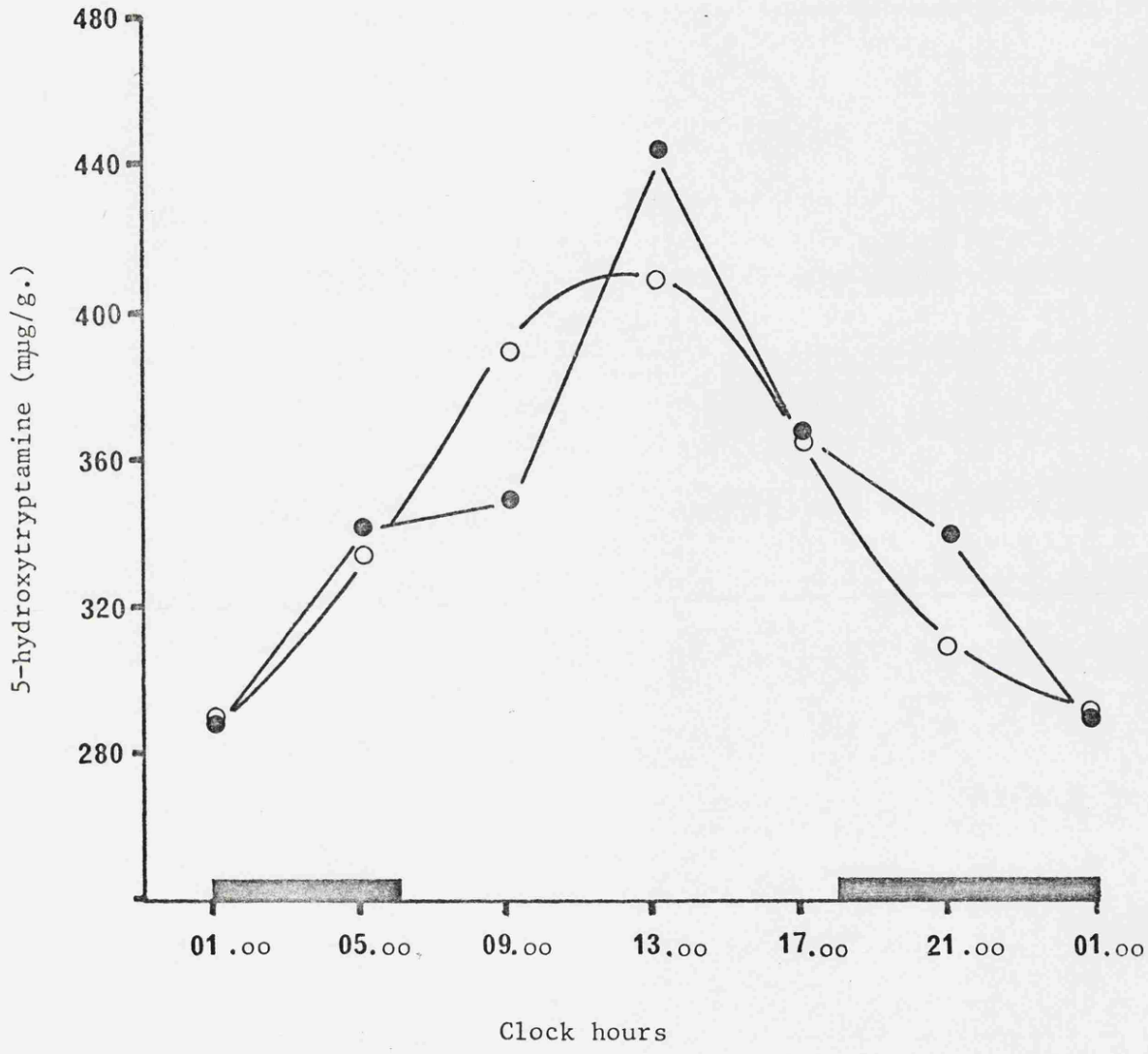


Figure 9. The twenty-four hour rhythm of 5-hydroxytryptamine concentrations (●——●) compared with the calculated sine curve (○——○).

respectively. The possible significance of this finding will be discussed in Chapter Twelve.

Because seven groups of animals were measured in the full twenty-four hours, animals in the first and last group were killed at the same clock hour on consecutive days. Since brain 5-hydroxytryptamine concentrations, determined from these two groups, did not show any significant difference, it can be concluded that the methods used were consistent. The twenty-four hour rhythm recorded is a true reflection of cerebral changes of 5-hydroxytryptamine, and is not an artifact dependent on environmental or operant variation.

The calculation of recovery from the extraction procedure was carried out by the use of internal standards and by istopic methods. The recovery was $48.4 \pm 1.6\%$. In these and all subsequent graphs and tables, values have not been corrected for recovery.

3.4 The in vivo and in vitro Uptake of 5-hydroxytryptamine into rat brain.

In vitro studies have demonstrated active transport mechanisms for the uptake of 5-hydroxytryptamine by blood platelets (Hughes and Brodie 1959; Weissbach, Redfield and Titus 1960), and by rat brain slices (Blackburn, French and Merrills 1967). Conversely Schanberg (1963) using rat brain, and Robinson, Anderson and Green (1965) using particulate fractions of rat brain, suggested that, in vitro, uptake of 5-hydroxytryptamine was due to passive diffusion and non-specific binding, although, in vivo, the uptake of 5-hydroxytryptamine by rat brain is affected by certain drugs (Palaic, Page and Khairallah 1967). Robinson, Anderson and Green (1965) indicated a non-specific ion-exchange binding of 5-hydroxytryptamine to synaptosomes and microsomal fractions of brain slices at a concentration of 1×10^{-4}M. 5-hydroxytryptamine has also been shown to bind to mitochondria and proteins at 0^{o}C (Marchbanks 1966), and to interact with lipid components of homogenates (Woolley and Campbell 1960).

More recently however, an active transport system has been shown to be involved in the uptake of 5-hydroxytryptamine into the brain of rats, mice and rabbits. Ross and Renyi (1969), investigating the uptake of ^{3}H-5-hydroxytryptamine into slices of mouse brain, found an active transport system, with K_m 7×10^{-7}M and V_{max} 0.23 mμ mole/g^{-1}/min^{-1}, which was limited to low concentrations, being masked by passive diffusion at higher concentrations (10^{-5}M). The uptake process could be blocked by tertiary tricyclic anti-depressants (imipramine, amitryptyline), rather less by secondary derivatives (desipramine, nortryptyline) and by cocaine both in vitro and in vivo. α-Alkylated tryptamines were equipotent with imipramine in blocking

5-hydroxytryptamine uptake. Carlsson (1970) calculated potency ratios for the blockade of the uptake process, with chlorimipramine > imipramine > desipramine.

Shaskan and Snyder (1970) proposed two processes of 5-hydroxytryptamine uptake, one of high affinity and one of low affinity, yielding K_m 1.7 x 10^{-7}M and 8 x 10^{-6}M respectively in the corpus striatum. They proposed the high affinity process to be the uptake of the amine into 5-hydroxytryptamine-containing nerve endings, consistent with the localisation of ^{3}H-5-hydroxytryptamine in these nerve endings following intra-ventricular injection of small doses of the ^{3}H-amine found by Aghajanian and Bloom (1967). The low affinity process was suggested to be the uptake of 5-hydroxytryptamine into catecholaminergic neurones since the K_i for competitive inhibition of noradrenaline uptake by 5-hydroxytryptamine approximately equalled the K_m for the low affinity uptake process Shaskan and Snyder (1970). Iversen (1970) showed that impairment of catecholaminergic function by the injection of 6-hydroxydopamine inhibited the low affinity process, while the high affinity process was not affected, thus supporting the view of Shaskan and Snyder (1970). Wong, Horng and Fuller(1973)similarly demonstrated two uptake processes with K_m 1 x 10^{-7}M and 7.91 x 10^{-6}M for the high and low affinity processes respectively.

Blockade of the uptake mechanisms has been shown by Horn (1973) using substituted derivatives of 5-hydroxytryptamine. Acetylation of the terminal amino group, methylation of the 5-hydroxyl group or substituting hydroxyl groups onto other positions reduced the affinity for the 5-hydroxytryptamine uptake site. Substitution of the tryptamine side chain in the α -position by methyl or ethyl but

not carboxyl groups, or increasing the side chain by one carbon atom increased the affinity for the uptake site. Further work by Horn, Baumgarten and Schlossberger (1973) on the substituted hydroxyl derivatives showed 5, 6-dihydroxytryptamine to be the most potent inhibitor of 5-hydroxytryptamine uptake.

A circadian variation has been shown for the uptake of 5-hydroxytryptamine in the hippocampus of rat brain (Wirtz-Justice and Hackmann 1973), but they were unable to find such a rhythm in other brain regions. Highest concentrations of 5-hydroxytryptamine were found at 08.00 h. and lowest concentrations at 20.00 h. on a lighting regimen 06.00 - 18.00 h.

Schubert (1973) summarised the regulating factors upon which 5-hydroxytryptamine uptake depends.

"The accumulation of ^{3}H-5-hydroxytryptamine in central neurones is dependent on the specific activity of the precursor, the rate of 5-hydroxytryptamine synthesis and the degree of retention of the newly formed amine by the endogenous stores".

3.5 The Uptake of ^{14}C-5-hydroxytryptamine into Homogenates of Rat Whole Brain.

3.5.1 METHODS

Male Sprague Dawley rats (120 - 140g) were housed under constant environmental conditions as described in Chapter Two for at least ten days before each experiment.

The animals were killed by decapitation, the pineal discarded, and the brain removed as previously described in this Chapter. The brain was immediately weighed and transferred to a glass homogeniser tube containing 10 ml. ice-cold 0.32M sucrose, and homogenised on ice using six strokes of a teflon pestle (0.01" clearance) at mark 4 on a Camlab K41 homogeniser. The homogenate was centrifuged at 500g at 4^{o}C for ten minutes. 1.0 ml. of the supernatant was added to 3.0 ml. Krebs-Ringer solution pH 7.4 of composition, NaCl 6.92, $MgSO_4.7.H_2O$. 0.144, KH_2PO_4 0.162, $NaHCO_3$ 2.1, KCl 0.354, $CaCl_2.6.H_2O$ 0.28, D-Glucose 2.0 (all values expressed as grams/litre distilled water) and pre-incubated at 37^{o}C for ten minutes. All solutions were made up in Krebs-Ringer solution pH 7.4. 1.0 ml. 5-hydroxytryptamine-3-^{14}C creatinine sulphate 57 mCi/m.mole (concentrations of the isotope, and conditions varying from those described here, are shown in the legends to the appropriate Figures) was added to the homogenate and incubated for varying times as indicated. 3.0 ml. ice-cold buffer was added and the homogenate centrifuged at 45,000g for twenty minutes at 0^{o}C. The supernatant was discarded, the pellet washed with buffer and the tube blotted dry. After the addition of 1.0 ml. hyamine hydroxide, the pellet was left

to digest overnight in the dark. 0.5 ml. of the digest was added to 5 ml. Unisolve (Koch Light), and counted in a Phillips PW4510/01 liquid scintillation analyser, thus allowing the uptake of ^{14}C-5-hydroxytryptamine to be determined.

3.5.2 The twenty-four hour Variation of 5-hydroxytryptamine Uptake

Since the variation of 5-hydroxytryptamine concentrations in the rat brain is so consistent with respect to clock hour, and the difference between nadir and zenith is so marked, it was considered justified, when investigating factors which may contribute to the twenty-four hour rhythm, to carry out estimations at the two clock hours when 5-hydroxytryptamine concentrations were highest and lowest, namely 01.00 h. and 13.00 h.

The effects of temperature, pH, incubation time, glucose concentration and the subcellular distribution and Michaelis constant were therefore determined at these two clock hours.

3.5.3 The Effect of Temperature on the Uptake of ^{14}C-5-hydroxytryptamine

The method used was as previously described, using a Krebs-Ringer solution pH 7.4. 1.0 ml. ^{14}C-5-hydroxytryptamine 1 x 10^{-7}M was added to the diluted homogenate and incubated for ten minutes at 4°C, 10°C, 22°C, 37°C, 45°C and 60°C.

3.5.4 The Effect of pH on the Uptake of ^{14}C-5-hydroxytryptamine

The Krebs-Ringer solutions used for the dilution of the homogenate, and for making up the ^{14}C-5-hydroxytryptamine solution were altered by the use of 2N-hydrochloric acid or 2N-sodium hydroxide to the following pH:- 5.0, 6.0, 7.0, 7.4, 7.6, 7.9, 9.0. 1.0 ml. of ^{14}C-5-hydroxy-tryptamine 5 x 10^{-7}M was added and incubated for ten minutes at 37°C.

3.5.5 The Effect of Incubation time and D-Glucose concentration on the Uptake of ^{14}C-5-hydroxytryptamine

1.0 ml. ^{14}C-5-hydroxytryptamine 5 x 10^{-7}M was incubated in Krebs-Ringer solution pH 7.4 at 37°C for the following times:- 15s, 45s, 75s, 1 min.45s, 2 mins.30s, 5 mins., 10 mins., 20 mins., 30 mins., 45 mins., 60 mins.,

The experiment was repeated using Krebs-Ringer solutions containing 1.0g/l D-Glucose, 0.25g/l D-Glucose and 0.0g/l D-Glucose instead of the normal 2.0g/l D-Glucose.

3.5.6 The Subcellular localisation of ^{14}C-5-hydroxytryptamine following Uptake into a homogenate of rat brain

The procedure for determining the uptake of ^{14}C-5-hydroxytryptamine was carried out exactly as described previously, using 1.0 ml ^{14}C-5-hydroxytryptamine 5 x 10^{-7}M in Krebs-Ringer solution pH 7.4 at 37°C for ten minutes. The subcellular localisation of ^{14}C-5-hydroxy-tryptamine which had been taken up into the homogenate was determined as follows:

The homogenate was immediately poured onto an ice-cold discontinuous sucrose gradient of composition 0.32M, 0.8M, 1.2M sucrose . The gradients

were centrifuged at 22,500 r.p.m. at $0^{o}C$ for sixty minutes in a Beckman ultracentrifuge with SW.25.1 swing out rotor. The fractions were collected by puncturing the bottom of the tube and allowing the contents to drip into collecting tubes. 0.5 ml. digested pellet, or 0.5 ml. hyamine-treated supernatant fractions were taken for liquid scintillation analysis.

3.5.7 Determination of the Michaelis constant (K_m)

1.0 ml. ^{14}C-5-hydroxytryptamine of concentration $10^{-7}M$ to $10^{-6}M$, was incubated with the homogenate for ten minutes at $37^{o}C$ in Krebs-Ringer solution pH 7.4. Since some of the isotope was taken up by facilitated diffusion at low temperatures (Figure 10), duplicate samples were incubated at $0^{o}C$ for ten minutes and the uptake was measured, and subtracted from the uptake recorded at $37^{o}C$. This was the procedure for calculating uptake kinetics throughout this thesis.

The results were plotted according to the method of Eisenthal and Cornish-Bowden (1974) (Figures 14, 15).

3.5.8 RESULTS AND DISCUSSION

At both clock hours, the uptake of ^{14}C-5-hydroxytryptamine into a homogenate of rat brain increased rapidly from $4^{o}C$ to a maximum at $37^{o}C$. Lower rates of uptake were recorded at higher temperature (Figure 10).

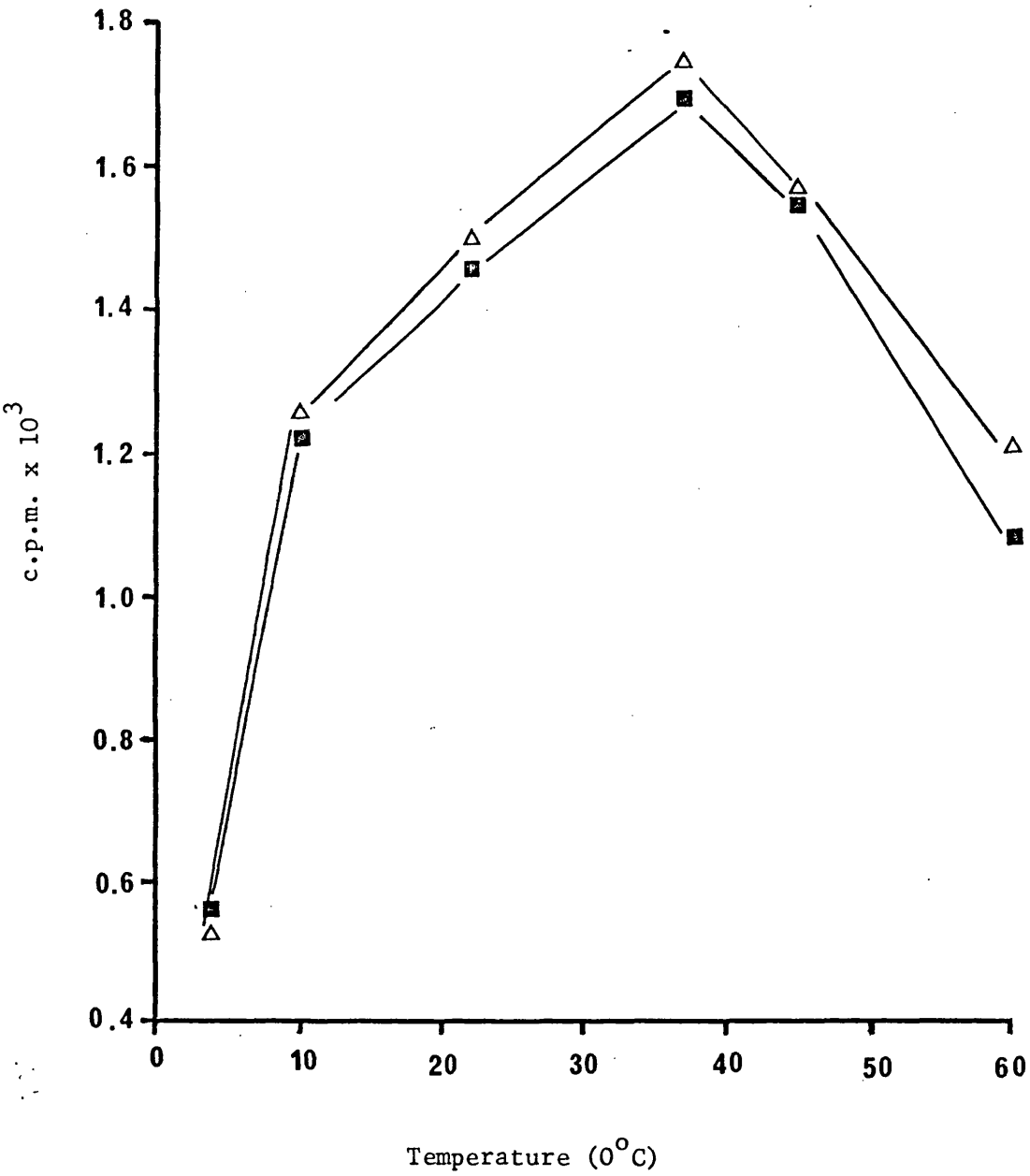


Figure 10. The effect of temperature on the uptake of ^{14}C-5-hydroxytryptamine ($1 \times 10^{-7}M$) into rat brain homogenates, at 01.00 h (■———■) and 13.00 h (△———△) at pH 7.4 following 10 minutes incubation.

A specific pH requirement was found (Figure 11), reaching maximum rates of uptake at pH 7.4 in Krebs-Ringer solution, and decreasing rapidly either side of this optimum. There was no significant difference between temperature and pH optima at the two clock hours measured.

The concentration of D-Glucose in the incubation medium was not critical, since even in a buffer containing no added D-Glucose, ^{14}C-5-hydroxytryptamine was readily taken up. At higher concentrations of glucose (1g/l and 2g/l), 30% and 32% increases respectively in the uptake of the amine were recorded. It would thus appear that while D-Glucose induced an increased uptake of ^{14}C-5-hydroxytryptamine when added to the incubation medium, there was sufficient glucose present in the homogenised brain to permit a limited uptake process.

The length of incubation time would appear to be important (Figure 12), since saturation of the uptake system occurred rapidly within the first two and a half minutes of incubation. The concentration of ^{14}C-5-hydroxytryptamine taken up in the homogenate was maintained only for the first ten minutes of incubation and was reduced after this period, possibly due to metabolism by monoamine oxidase, although Kannengiesser, Hunt and Raynaud (1973) found that 85% of the amine taken up was not metabolised.

The initial rapid accumulation of ^{14}C-5-hydroxytryptamine indicated that the uptake measured was not a single process. Since the method made measurement of uptake extremely difficult at times less than 15s., this part of the process has not been further investigated. 33% of the amine was taken up in this initial 15s. period, a rate more than four times greater than that during the following two minutes. It seems unlikely from published data on uptake kinetics that this could be due to an

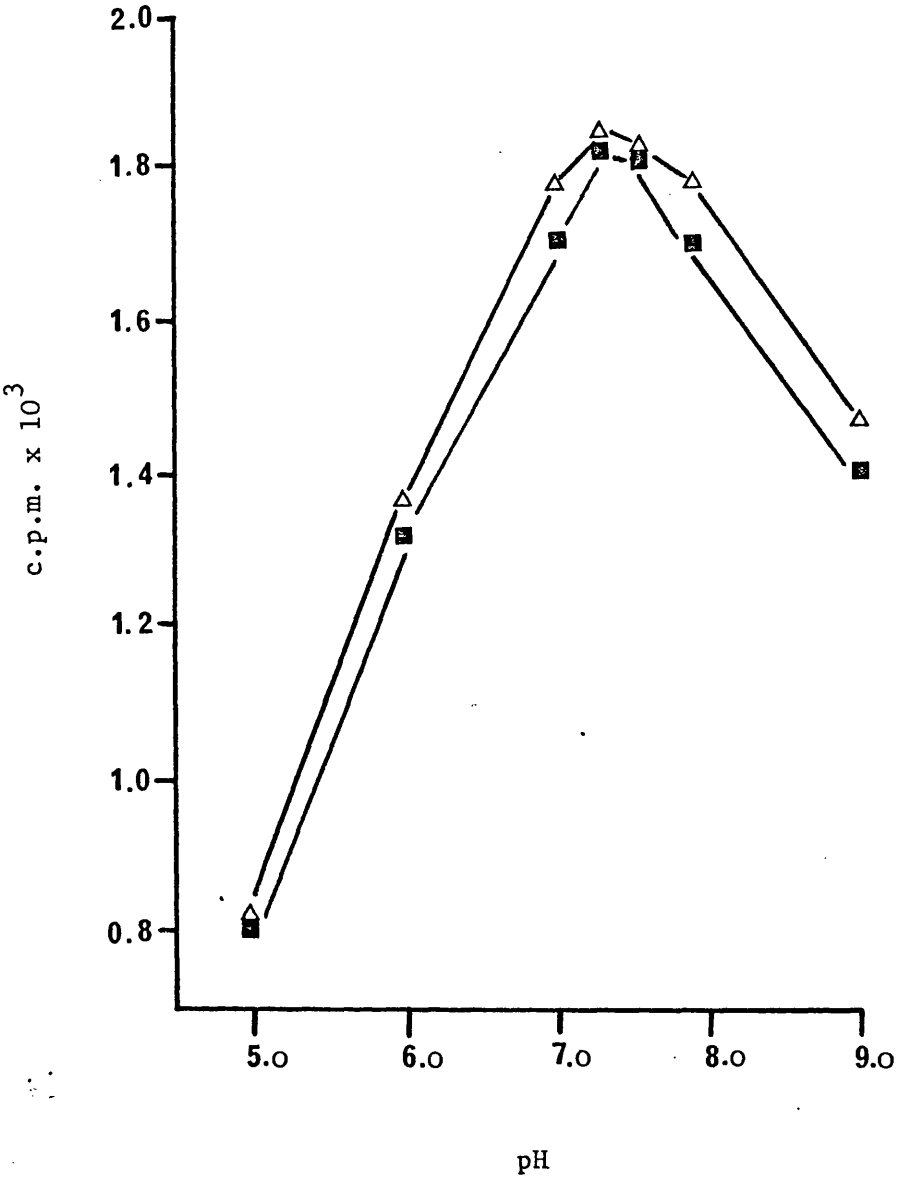


Figure 11. The effect of pH on the uptake of ^{14}C-5-hydroxytryptamine (5 x 10^{-7}M) into rat brain homogenates at 01.00 h (■——■) and 13.00 h (△——△) at 37°C following 10 minutes incubation.

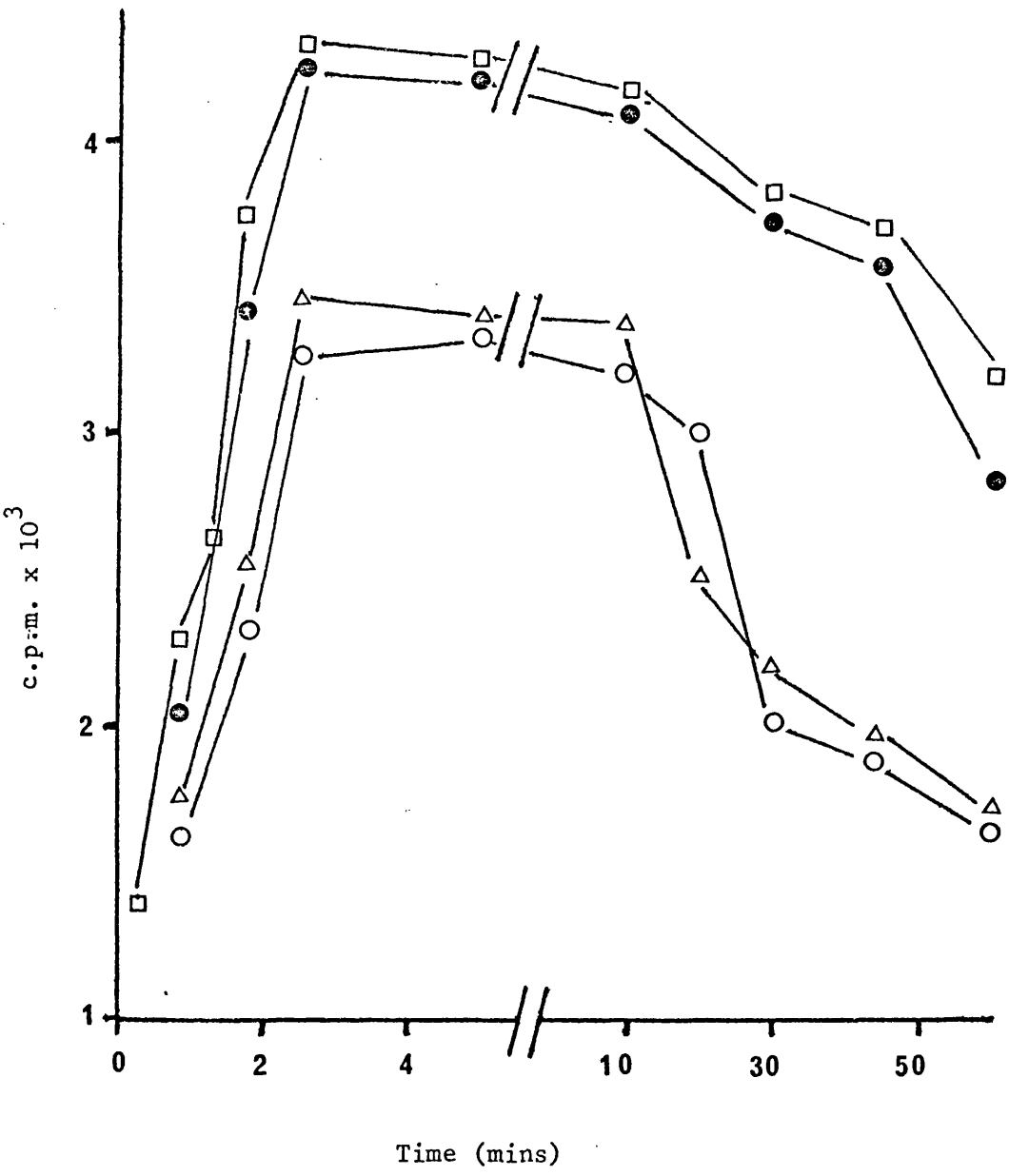


Figure 12. The effects of incubation time and glucose concentration on the uptake of ^{14}C-5-hydroxytryptamine (5 x 10^{-7}M) into homogenates of rat brain at 13.00 h. Glucose concentrations 0g/l (O—— O), 0.25 g/l (△—— △), 1.0 g/l (● —— ●), 2.0 g/l (□ —— □), pH 7.4 and temperature 37°C.

initial very rapid uptake process. Since passive diffusion accounts for only a small proportion of the amine taken up, the most plausible explanation would appear to be that the amine exhibits a high degree of non specific binding. This binding apparently occurred in the nerve ending fraction, since 64% of the total radioactivity was found in this fraction, with the remainder in the supernatant (Figure 13), and little associated with other particulate fractions.

The effects of incubation time and D-Glucose concentration were similar at the two clock hours measured.

The rate of uptake of ^{14}C-5-hydroxytryptamine was not proportional to the concentration of exogenous substrate. A carrier-mediated transport process of limited capacity is thus implicated. The requirement for specific temperature and pH optima and the increase in uptake with D-Glucose concentration indicated an energy-requiring process. The K_m for ^{14}C-5-hydroxytryptamine uptake was 2.5×10^{-6}M at 13.00 h. and 3.1×10^{-6}M at 01.00 h. (Figures 14, 15). These two estimates were not significantly different. When calculated as c.p.m./g^{-1}/5 min., V_{max} was approximately 15% higher at 01.00 h. than at 13.00 h. (Figures 14, 15). This calculation does not allow for the difference in specific activity at the two clock hours. The implications of this will be discussed further at the end of this Chapter.

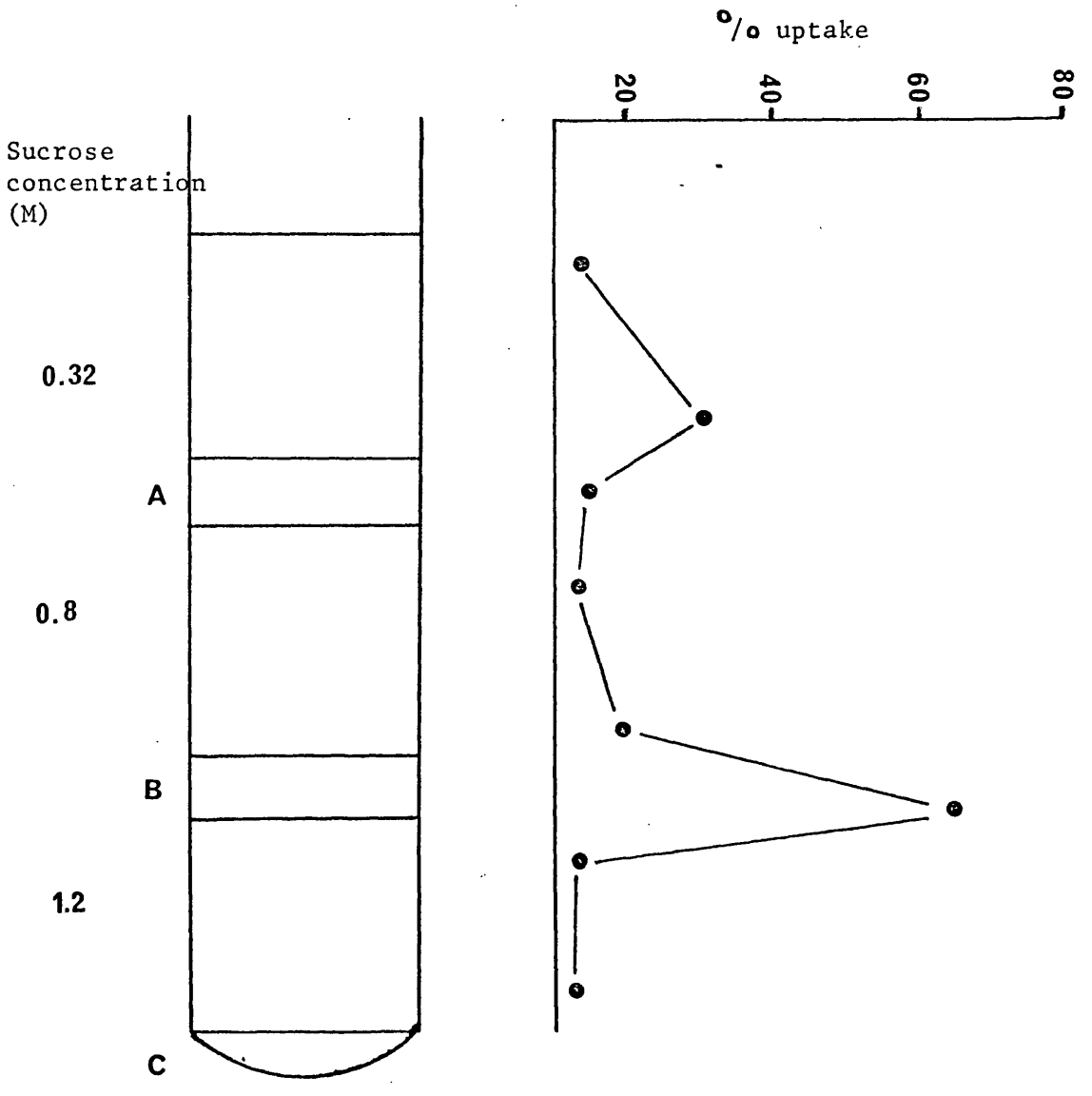


Figure 13. The subcellular distribution of the uptake of ^{14}C-5-hydroxytryptamine (5×10^{-7}M) at 37°C pH 7.4. A-myelin component; B-nerve endings; C-mitochondria. The percentage uptake in each component is shown in the accompanying graph. Incubation time was 10 minutes.

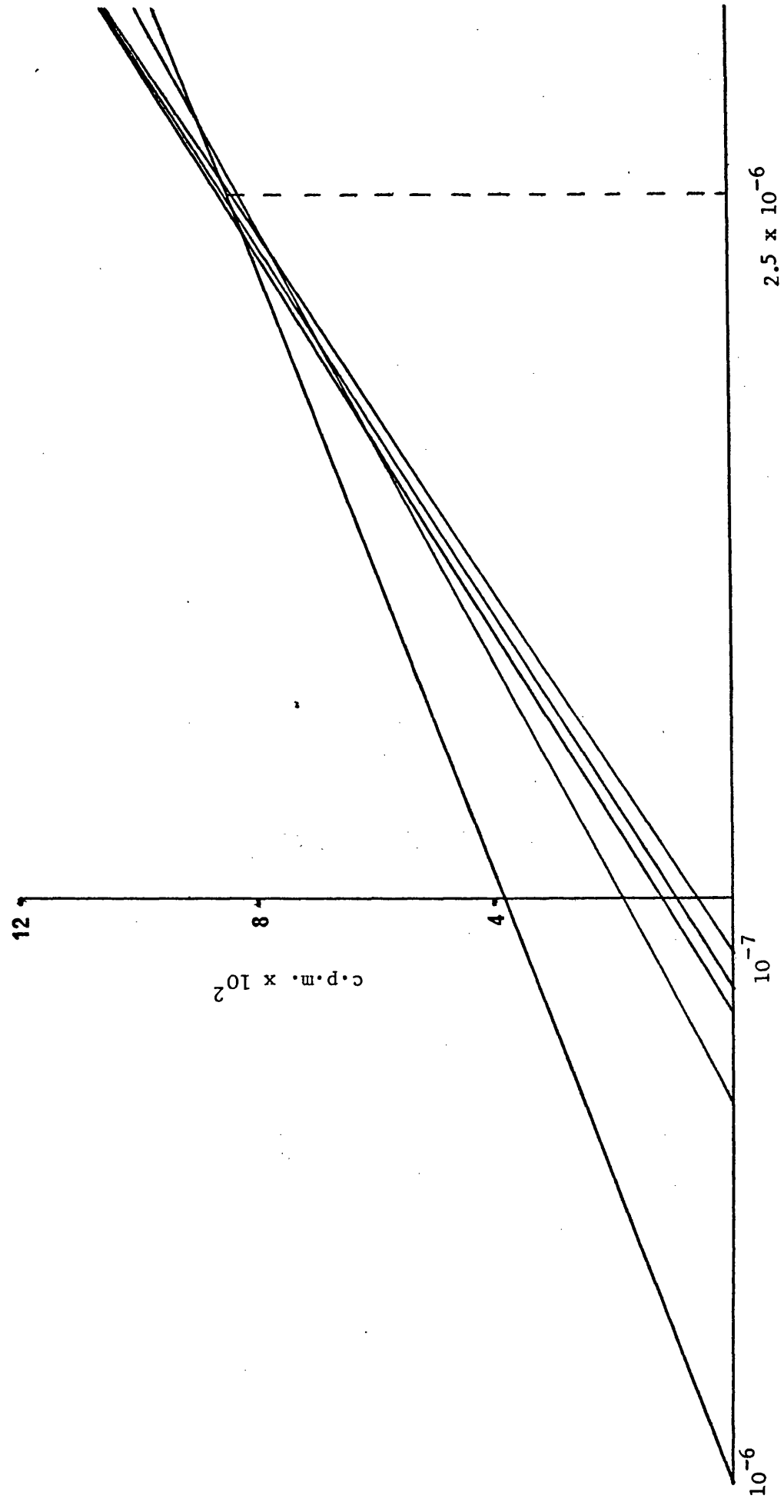


Figure 14. Direct linear plot to determine the Michaelis constant of ^{14}C-5-hydroxtryptamine uptake into rat brain homogenates at 13.00 h. at 37°C and pH 7.4.

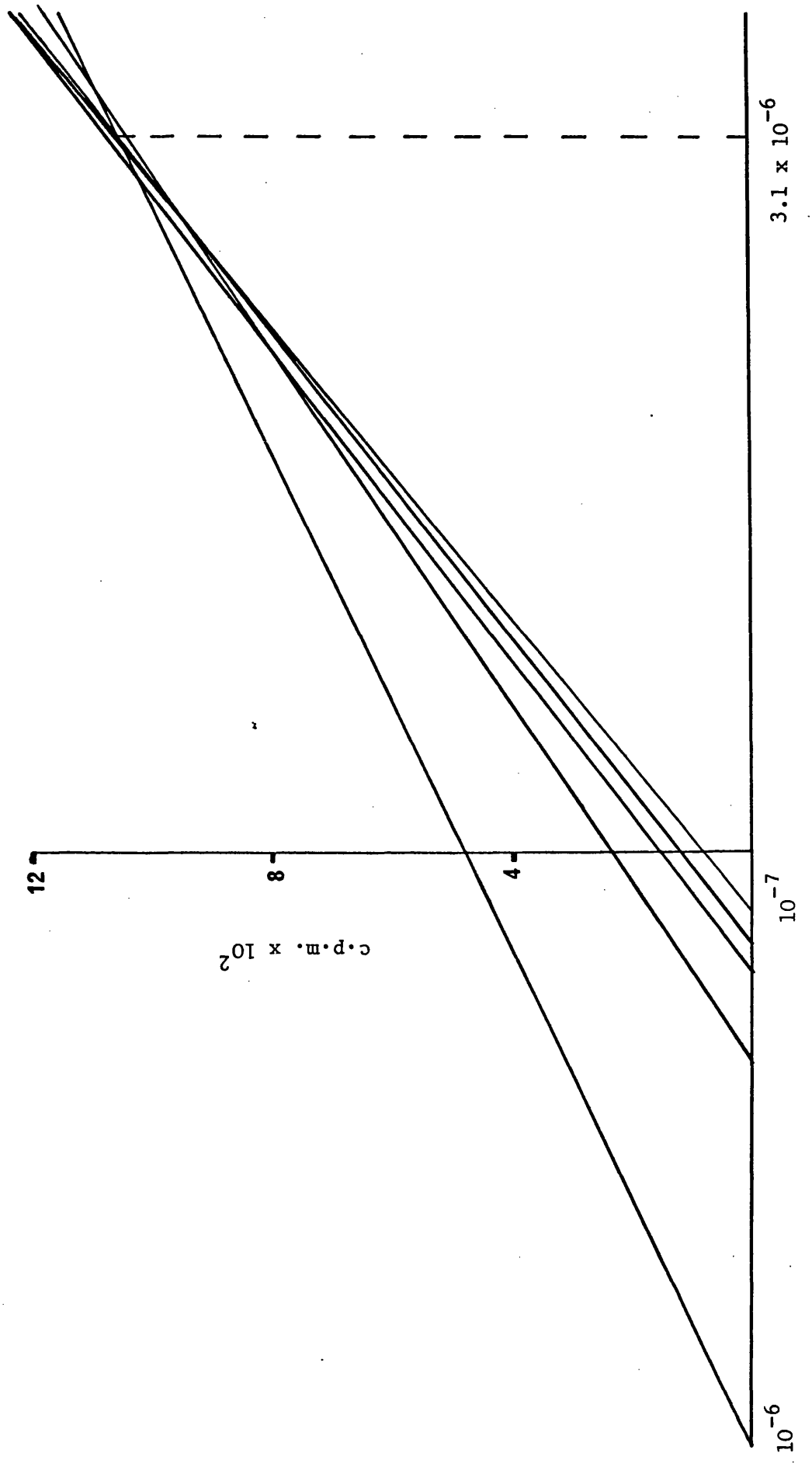


Figure 15. Direct linear plot to determine the Michaelis constant for the uptake of ^{14}C-5-hydroxytryptamine into rat brain homogenates at 01.00 h. at 37°C and pH 7.4.

3.6 The Uptake of ^{14}C-5-hydroxytryptamine into Homogenates of Rat Brain Regions

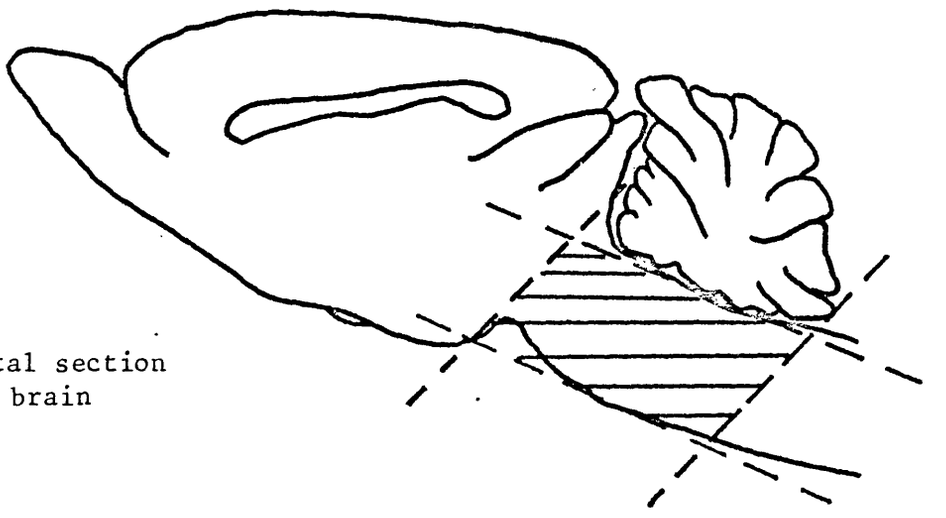
3.6.1 METHODS

The rats were killed and the brains removed as described previously.

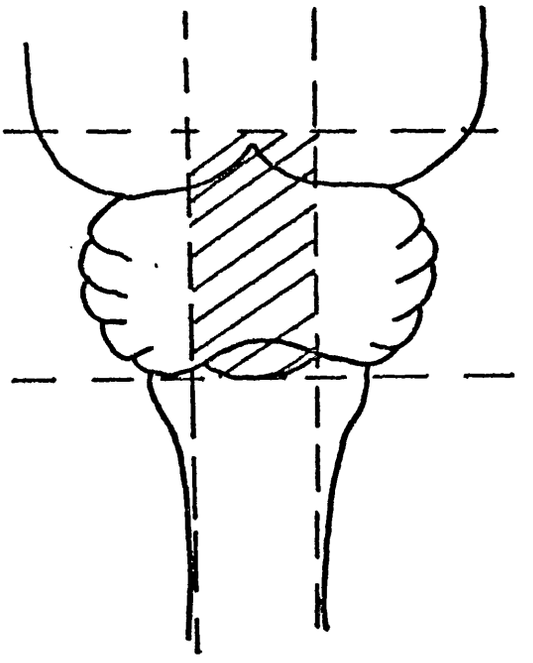
The greatest concentrations of 5-hydroxytryptamine-containing nerve endings are in the septal region and the suprachiasmatic nucleus, and the cell bodies of these nerve endings are found in the raphe nuclei (Chapter One). The region containing the septum and the region containing the raphe nuclei were therefore taken for the estimation of the uptake of ^{14}C-5-hydroxytryptamine.

The brain stem area containing the raphe nuclei was dissected out (Figure 16) and immediately homogenised in 2.0 ml. ice-cold Krebs-Ringer solution pH 7.4. The pestle and tube were washed in a further 2.5 ml. ice-cold Krebs-Ringer solution and the washings added to the homogenate. The homogenate was diluted to 7.5 mls. with ice-cold Krebs-Ringer solution and kept on ice.

The septal region was removed (Figure 17) from two rats and homogenised separately in 1.0 ml. ice-cold Krebs-Ringer solution pH 7.4. The homogeniser pestle and tube were washed with 2.5 ml. ice-cold Krebs-Ringer solution and the washings added to the homogenate. The two homogenates were combined and the whole made up to 7.5 ml. with ice-cold Krebs-Ringer solution and kept on ice. The weight of tissue in the final homogenate was approximately 30 mg.



saggital section
of rat brain



dorsal view

Figure 16. Diagrammatic representation of the area removed (shaded area) and used as a source of nerve cell bodies of 5-hydroxytryptamine-containing neurones. (--- represents the lines of cut).

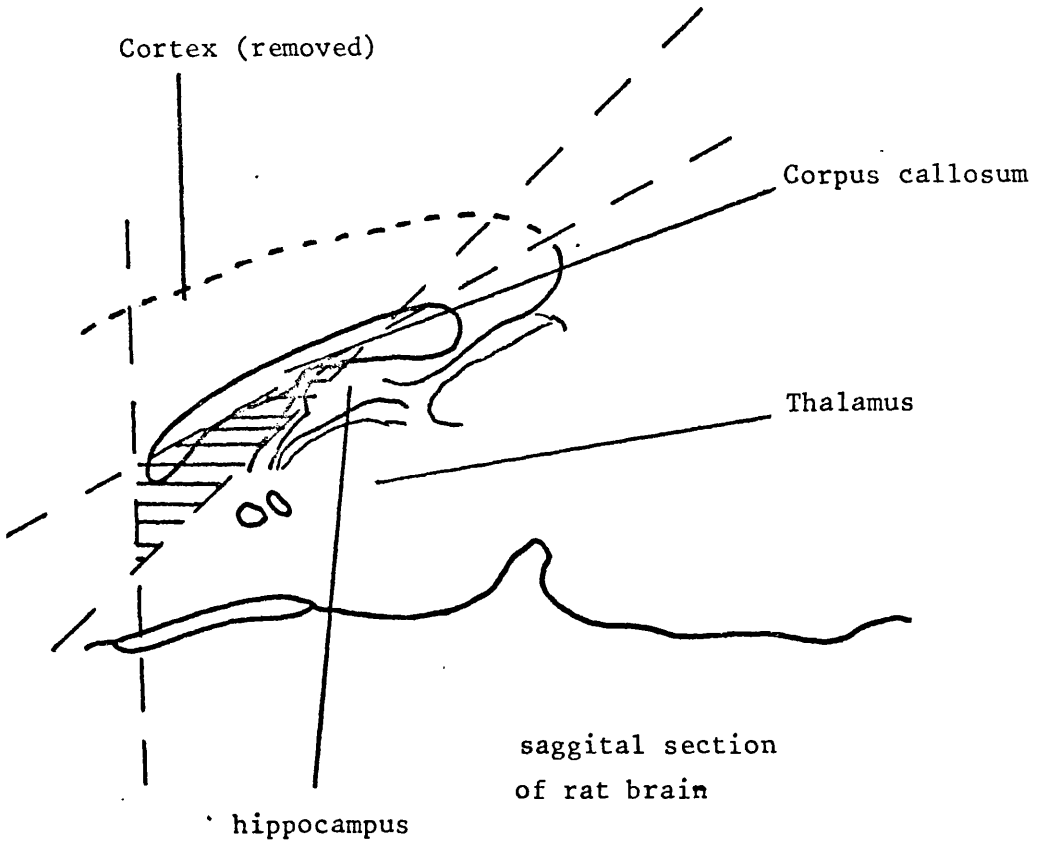


Figure 17. Diagrammatic representation of the area removed (shaded) and used as a source of nerve endings from 5-hydroxytryptamine-containing neurones. (--- represents the lines of cut).

3.6.2 Estimation of the Michaelis Constant (K_m)

The homogenates containing raphe nuclei and septal regions were incubated for 5 mins. at 37°C to equilibrate. 2.5 mls. of a solution containing ^{14}C-5-hydroxytryptamine in Krebs-Ringer solution was added. Concentration of the isotope are shown in Figures 18, 19, 20, 21. 1.0 ml. samples of the incubation mixture were taken after 1,2,4,6,8 and 10 mins. and immediately passed through a 0.22μ millipore filter. The residue was washed with 5 ml. ice-cold Krebs-Ringer solution to remove excess isotope. The filters were blotted dry, immersed in 5 ml. Unisolve and taken for liquid scintillation analysis as before. The Michaelis constant was estimated at 01.00 h. and 13.00 h. as previously described in this Chapter. The results are the means of three determinations.

3.6.3 RESULTS AND DISCUSSION

The Michaelis constant of the septal region was estimated as 4.9×10^{-7}M at 13.00 h. and 5.8×10^{-7}M at 01.00 h. Figures 18,19. Since there is no significant difference between these two figures it can be concluded that there is no twenty-four hour variation in the K_m for the uptake of ^{14}C-5-hydroxytryptamine into nerve endings of the septal region of the brain of rats.

The Michaelis constant of the uptake of ^{14}C-5-hydroxytryptamine into a homogenate of brain stem was estimated as 1.2×10^{-6}M at 01.00 h. and 1.3×10^{-6}M at 13.00 h. Figures 20, 21. These results do not suggest a twenty-four hour variation of the K_m for uptake of the amine in this region. These estimations are similar to those for whole brain, but are less than those for the septal region, although the difference is not sufficiently large to suggest a different mechanism for the uptake of the amine in this region.

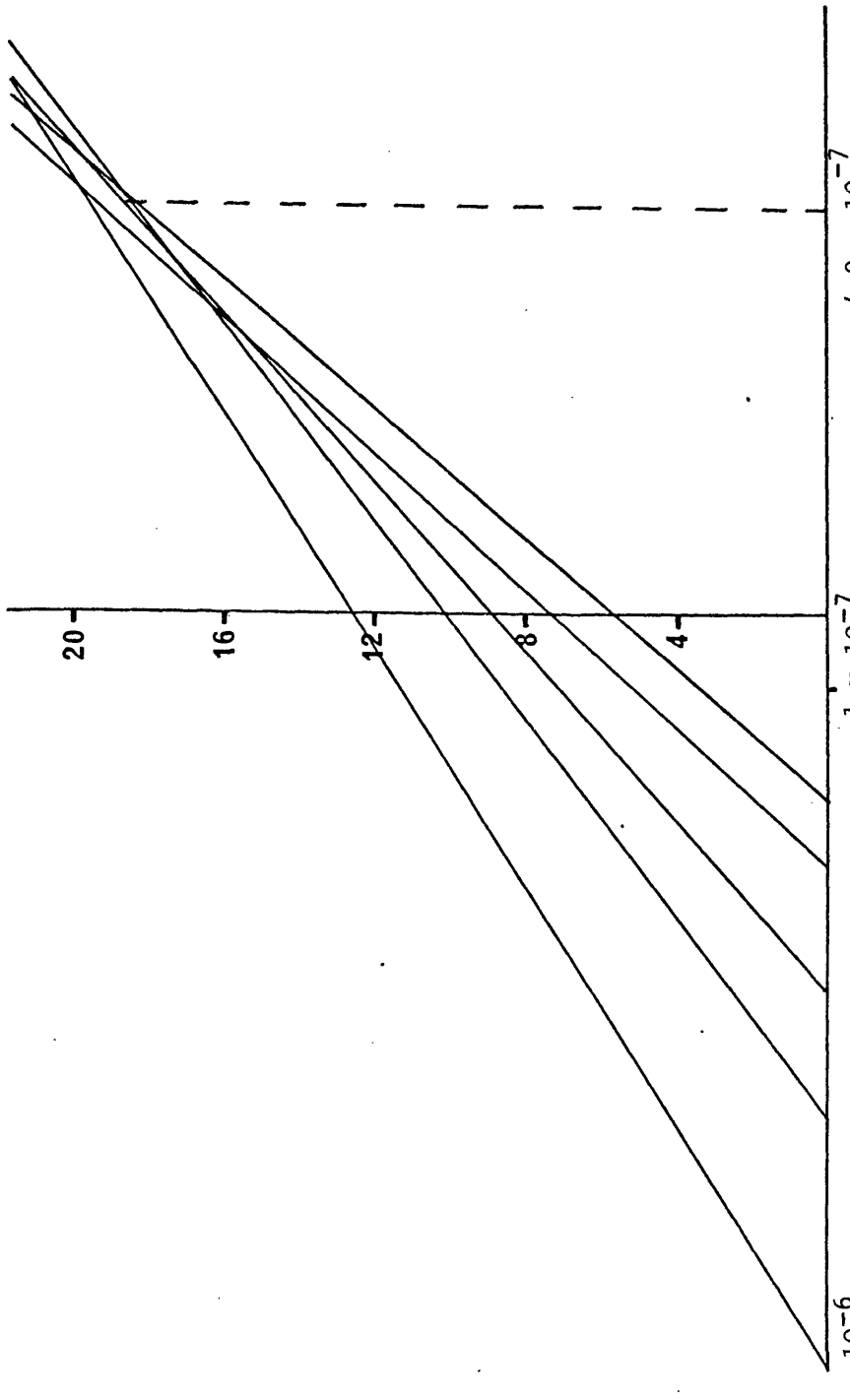


Figure 18. Direct linear plot to determine the Michaelis constant of ^{14}C-5-hydroxytryptamine uptake into homogenates of the septal region of rat brain at 13.00 h. at 37°C and pH 7.4.

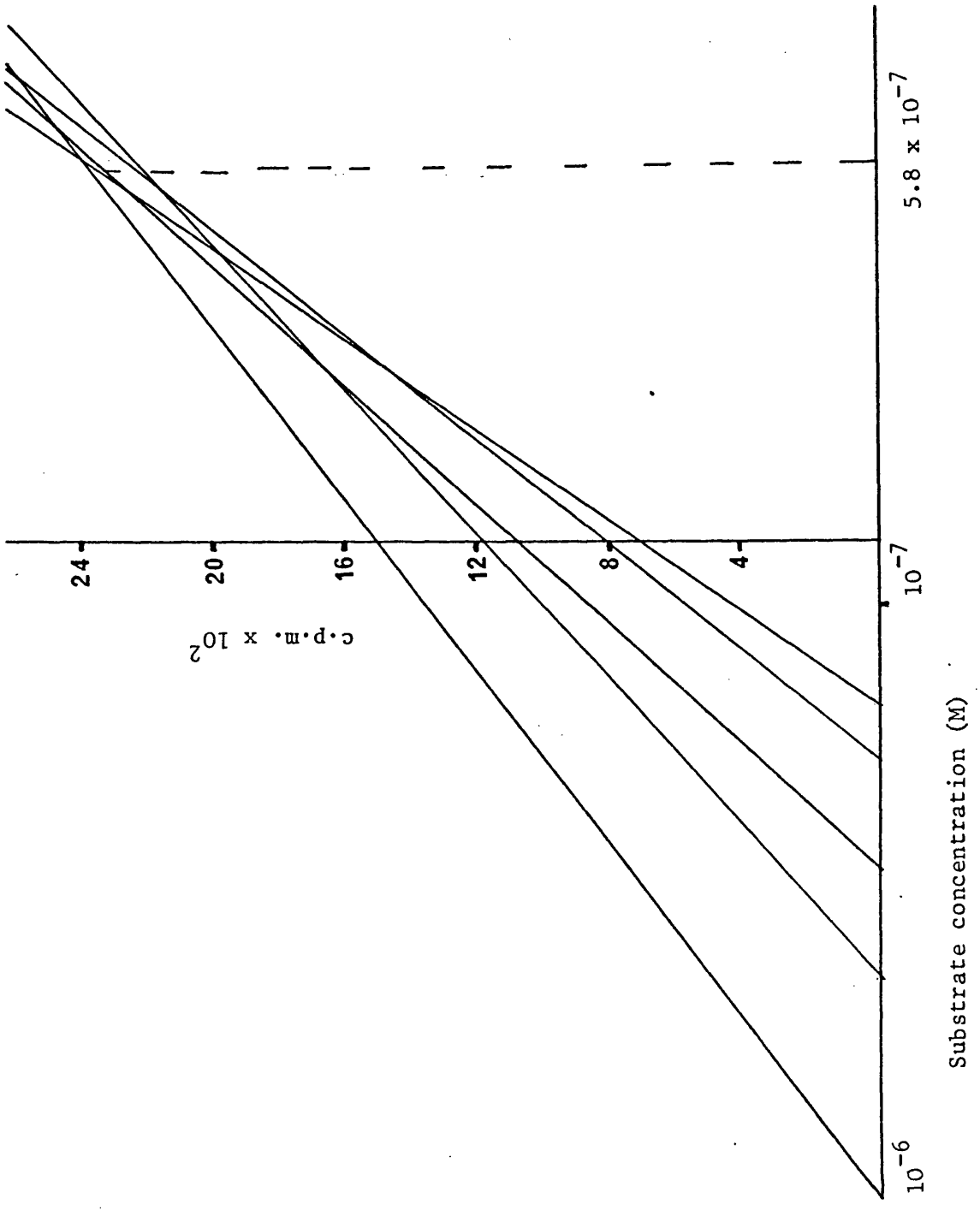


Figure 19. Direct linear plot to determine the Michaelis constant of ^{14}C-5-hydroxytryptamine uptake into homogenates of the septal region of rat brain at 01.00 h. at 37°C and pH 7.4.

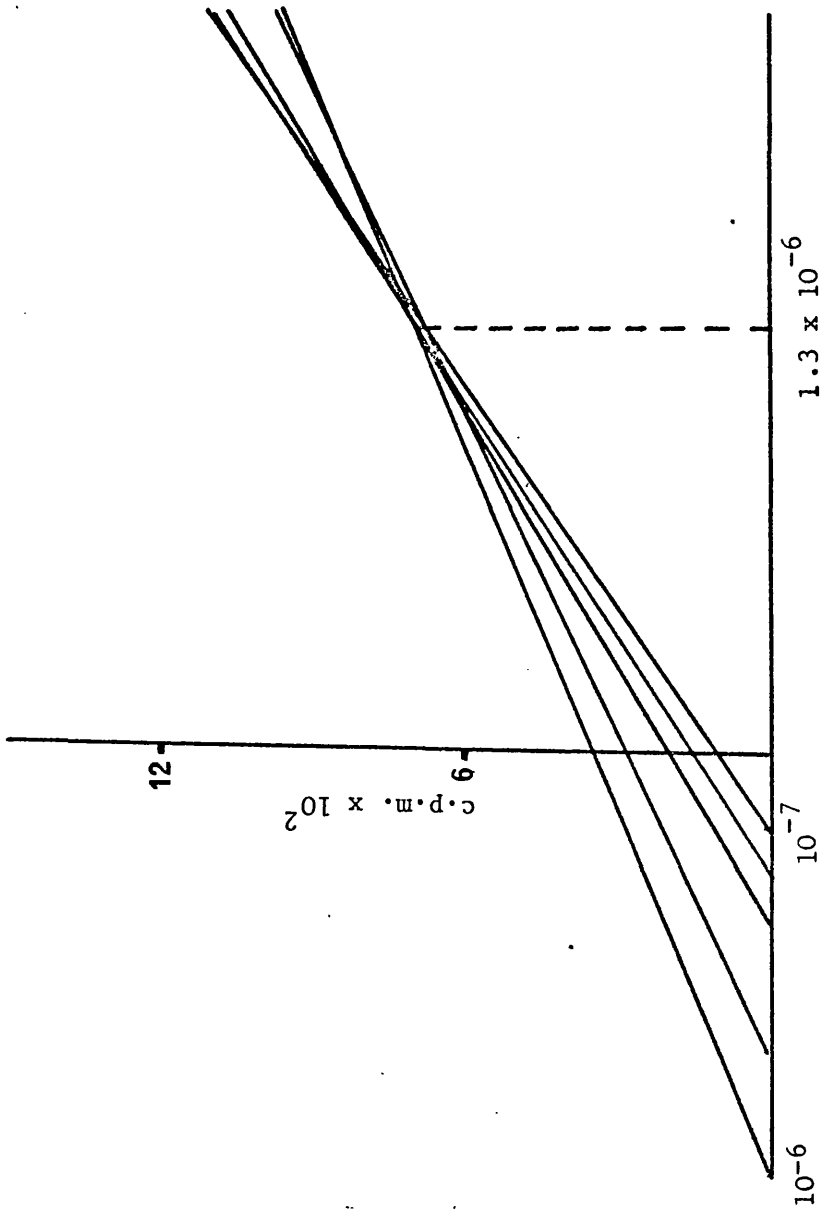


Figure 20. Direct linear plot to determine the Michaelis constant for the uptake of ^{14}C-5-hydroxytryptamine into homogenates of brain stem at 01.00 h. at 37^{0}C and pH 7.4.

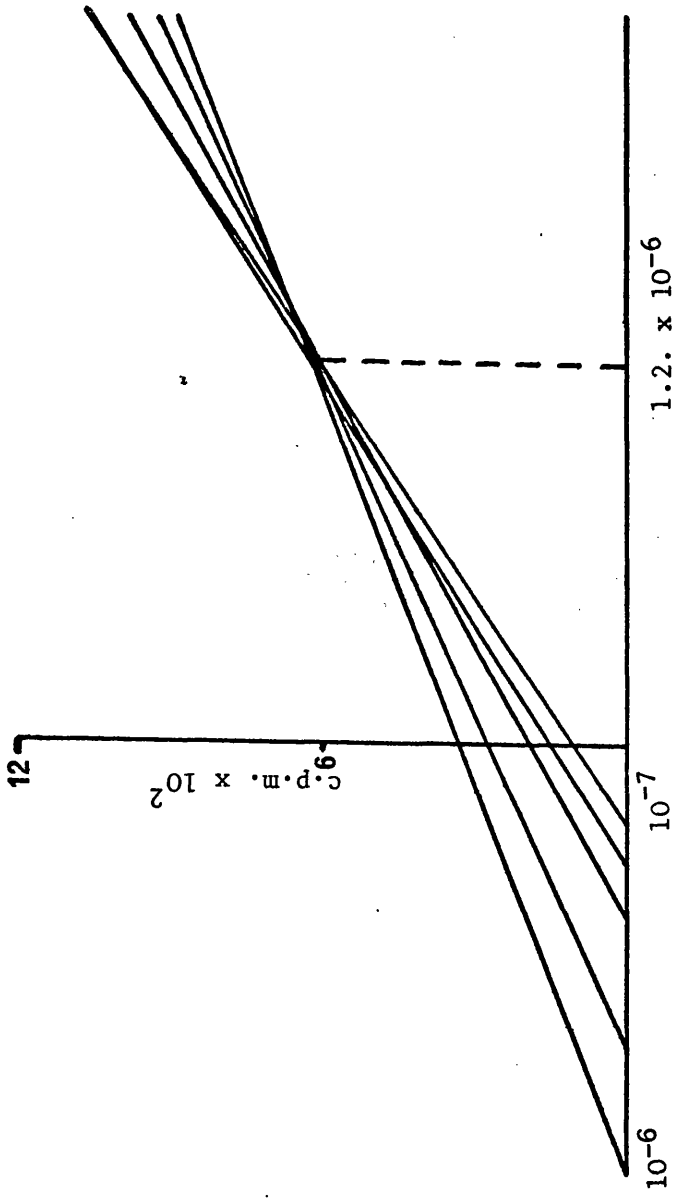


Figure 21. Direct linear plot to determine the Michaelis constant for the uptake of ^{14}C-5-hydroxytryptamine into homogenates of brain stem at 13.00 h. at 37°C. and pH 7.4.

Since it has already been shown that there is a twenty-four hour rhythm of 5-hydroxytryptamine concentrations in the rat brain, it is apparent that there will be different concentrations of 5-hydroxytryptamine in the homogenates taken at different clock hours. The specific activity of the ^{14}C-5-hydroxytryptamine in these homogenates will therefore be different. This will not affect the K_m values calculated, but it will affect V_{max}. The values of V_{max} shown in Figures 14, 15, 18, 19, 20 and 21 have been calculated without taking the different endogenous concentrations of 5-hydroxytryptamine into account. When these values are recalculated taking into account the different endogenous concentrations of 5-hydroxytryptamine, the samples taken at 13.00 h, which previously showed V_{max} to be approximately 14% lower than those taken at 01.00 h, now show V_{max} to be approximately 12% higher than those taken at 01.00h. V_{max} calculated by this method still did not differ significantly between the two clock hours, so it must be concluded that V_{max} does not display rhythmic variations in relation to the twenty-four hour day.

CHAPTER FOUR

The Role of Tryptophan in the Twenty-four Hour Variation of 5-hydroxytryptamine concentrations.

4.1 INTRODUCTION

Tryptophan, an essential amino acid, is the necessary precursor of brain 5-hydroxytryptamine (Udenfriend, Clark and Titus 1953; Udenfriend, Titus, Weissbach and Peterson 1956). Dalgleish and Dutton (1957) proposed that tryptophan must be hydroxylated extra-cerebrally, and that cerebral synthesis of 5-hydroxytryptamine depends on the rapid entry of 5-hydroxytryptophan into the brain and its subsequent and immediate decarboxylation to 5-hydroxytryptamine. Grahame-Smith (1964) using an *in vitro* technique, and Gal, Poczik and Marshall (1963); Gal, Morgan and Marshall (1965); Gal, Morgan, Chatterjee and Marshall (1964); Weber and Horita (1965); and Weber (1966) working *in vivo* showed that cerebral tissue can hydroxylate tryptophan, but that the process was too slow to account for all cerebral 5-hydroxytryptamine. It was therefore thought that extra-cerebral hydroxylation of tryptophan must complement that which occurs in the brain.

In this Introduction I shall describe the details of the metabolism and distribution of the amino acid peripherally as well as in the CNS, and the relationship between these two systems. The effects of diet on tryptophan concentrations, and the effect of tryptophan concentration on the synthesis of 5-hydroxytryptamine will also be considered. Finally, evidence for the twenty-four hour variation of tryptophan concentrations, and evidence for a high affinity uptake process into nerve endings will be produced.

Further evidence that tryptophan is the necessary precursor of 5-hydroxytryptamine has been presented by Ashcroft, Eccleston and Crawford (1965). Using the technique of tryptophan loading, they showed that the intra-peritoneal injection of 800 mg/kg tryptophan caused a rapid rise in brain 5-hydroxytryptamine and

5-hydroxyindole-3-acetic acid concentrations. Wang, Harwalkar, and Waisman (1962) and Quay (1963) found increased brain 5-hydroxytryptamine concentrations simply by feeding a tryptophan supplemented diet to rats. Conversely Culley, Saunders, Mertz and Jolly (1963) fed a tryptophan deficient diet to rats and found plasma tryptophan and brain 5-hydroxytryptamine concentrations to be significantly decreased. Similar results were obtained by Zbinden and Pletscher 1958; Gal and Drewes 1962 and Quay 1963.

Yuwiler (1973) reported a lack of stereospecificity for the conversion of tryptophan to 5-hydroxytryptamine, although the D-isomer must undergo peripheral deamination and subsequent transamination to the L-form. The D-isomer is therefore slower acting and less potent.

Since it has been widely shown that the synthesis of 5-hydroxytryptamine in the CNS is dependent on the presence of the amino acid tryptophan, and since cerebral tryptophan supplies are dependent on circulating tryptophan in the blood, it is perhaps pertinent to examine the role of tryptophan peripherally.

Tryptophan enters the systemic circulation from two main sources i.e. overflow from the portal circulation following protein ingestion, and efflux from bound and free tryptophan pools in the tissues. Tryptophan is removed from the circulation by uptake into the tissues, including the brain, metabolism in the liver by tryptophan pyrrolase or by excretion in the urine.

The metabolism of tryptophan via the 5-hydroxytryptamine pathway accounts for only 1% of urinary tryptophan metabolites (Michael, Drummond, Doeden, Anderson and Good 1964). Curzon and Green (1969) confirmed that the major route of tryptophan metabolism starts with its oxidation to formyl kynurenine by tryptophan pyrrolase in the liver, and only a minor route leads to 5-hydroxytryptamine.

L-tryptophan is unique among amino acids in that it is bound to a non-dialysable plasma component (McMenamy, Lund and Oncley 1957; McArthur and Dawkins 1969) which was identified as serum albumin (McMenamy and Oncley 1958). Binding is strongest at the indole-protein bond, although bonding by the tryptophan carboxyl to protein and a 'close fit' to the protein at the hydrogen of the tryptophan which would not permit the amino group to occupy this position completes the bound structure. Approximately 20-25% of tryptophan remains unbound or free (McMenamy, Lund, van Marcke and Oncley 1961; McArthur and Dawkins 1969) although lowered pH releases the amino acid from its bound form. Johnson and Bergeim (1951) found a 3 or 4:1 plasma: erythrocyte ratio for tryptophan, although McMenamy, Lund, van Marcke and Oncley(1961),failed to confirm this ratio and concluded that tryptophan is evenly distributed in the blood.

Tryptophan plays a major role in peripheral metabolism other than in 5-hydroxytryptamine synthesis. The availability of plasma free tryptophan can be a limiting factor in hepatic protein biosynthesis (Munro 1968) and tryptophan facilitates the formation of polysomal aggregates in liver, and enhances protein synthesis (Sidransky, Sarma, Bongiurno and Verney 1968). Under certain conditions, release of adrenal corticoids in response to stress induces tryptophan pyrrolase which may lower tryptophan levels to such an extent that peripheral metabolism would be shifted from protein synthesis to gluconeogenesis while simultaneously lowering brain 5-hydroxytryptamine. Tryptophan has been reported to inhibit gluconeogenesis by converting and maintaining phosphoenolpyruvate carboxykinase, (EC.4.1.1.32.) in a non-functional state (Ray, Foster and Lardy 1966).

Yuwiler (1973) suggested that brain 5-hydroxytryptamine biosynthesis was controlled by its precursor, tryptophan. Fernstrom and Wurtman (1971) and Fernstrom, Larin and Wurtman (1973) proposed that the control of brain 5-hydroxytryptamine synthesis was coupled to plasma tryptophan levels. Conversely Curzon, Joseph and Knott (1972) found brain and plasma tryptophan levels not to be correlated, but Knott and Curzon (1972) found brain tryptophan and plasma free tryptophan to be associated, and Curzon, Friedel and Knott (1973) demonstrated that plasma non-esterified fatty acid changes have a role in determining the availability of tryptophan to the brain. This latter finding is in agreement with Fernstrom, Larin, and Wurtman (1973), who showed that brain tryptophan was increased by administering a carbohydrate and fat diet, although not when 18 -24% protein was added. The effect of diet was not well correlated with plasma tryptophan concentration alone, but did correlate with the ratio of plasma tryptophan to individual amino acids (Leucine, isoleucine, valine, tyrosine and phenylalanine) or to the sum of the concentration of those amino acids. Carbohydrate depressed neutral amino acids while elevating plasma tryptophan. Protein ingestion raised neutral amino acid levels and altered the balance for their competitive uptake against tryptophan into the brain (Fernstrom and Wurtman 1971). These workers also suggested that the serotonin-containing neurones provide the rest of the brain with information about a broad range of metabolic states, since a wide variety of dietary and hormonal inputs probably can influence the ratio of plasma tryptophan to its competitor amino acids.

The effects of non-esterified fatty acids on the plasma free tryptophan concentrations in plasma have been recently investigated by Lipsett, Madras, Wurtman and Munro (1973). Oleic acid added to serum increased the free tryptophan concentrations, whereas glucose ingestion (75 g. to humans) decreased the plasma free tryptophan by 35% with no change in total plasma tryptophan concentrations. Munro and Thompson (1953) found only a small decrease in total serum tryptophan concentrations, following carbohydrate ingestion, compared with the larger reduction in other amino acids. This decrease occurred in the non-albumin-bound fraction, and may be explained by either an insulin mediated increase in the transport of free amino acids into some tissues, notably muscle, or a reduction in non-esterified fatty acid by glucose administration reducing the availability of free tryptophan by increasing its binding to albumin. Albanese, Irby, Frankston and Larin (1947) recorded an immediate fall in urinary tryptophan following carbohydrate ingestion.

Drugs which increased the concentration of non-esterified fatty acids e.g. isoprenaline, aminophylline, heparin (Curzon and Knott 1973), also increased plasma free tryptophan concentrations. Aminophylline increased brain tryptophan, 5-hydroxytryptamine and 5-hydroxyindole-3-acetic acid in addition. Fatty acids increased free plasma tryptophan, while nicotinic acid prevented the increase in plasma free tryptophan induced by food deprivation, and also increased plasma non-esterified fatty acid concentrations. Insulin decreased non-esterified fatty acids and tryptophan concentrations. These results are consistent with a positive correlation between changes in tryptophan metabolism and adenosine-3',5'-monophosphate (cyclic AMP), as this is known to mediate the lipolytic action of the catecholamines. Aminophylline and nicotinic acid increased and decreased fat-cell cyclic AMP respectively. This

suggested that the disposition of plasma tryptophan and brain 5-hydroxytryptamine metabolism could be influenced by the hormonal factors (Robison, Butcher and Sutherland 1971) controlling extra-cerebral cyclic AMP and fatty acid production.

Tagliamonte, Tagliamonte, Forn, Perez-Cruet, Krishna and Gessa (1971(a)) demonstrated a marked increase in brain tryptophan concentrations induced by cyclic AMP and dibutyryl cyclic AMP. They assumed that the cyclic AMP system was located within nerve endings where monoamine containing vesicles were also present (De Robertis 1964). More recently Tagliamonte, Biggio,Vargiu and Gessa (1973(a)) concluded that brain tryptophan and serotonin turnover were controlled by free serum tryptophan, which is itself independent of total serum tryptophan.

Madras, Cohen, Fernstrom, Larin, Munro and Wurtman (1973) lent a word of caution to this interpretation of these findings. They recognised that the concentration of free tryptophan in serum did not regulate the changes in brain tryptophan concentrations that normally accompany such physiological occurrences as eating, although free tryptophan may be correlated with brain tryptophan after certain treatments such as drug administration (Tagliamonte, Biggio and Gessa 1971(c); Tagliamonte, Tagliamonte, Perez-Cruet and Gessa 1971(b)), or a prolonged fasting period or immobilisation stress (Knott and Curzon 1972) or electro convulsive shock (Tagliamonte, Tagliamonte, DiChiara, Gessa and Gessa 1972).

Perhaps of clinical importance is the finding that salicylate (McArthur and Dawkins 1969, Tagliamonte, Biggio,Vargiu and Gessa 1973) and other anti-inflammatory drugs (McArthur, Dawkins and Smith 1971) may be up to 90% bound to the albumin fraction, while tryptophan may consequently be virtually absent from the serum of humans being treated

with these drugs. Tryptophan has been shown to be metabolised via the kynurenine pathway under these conditions (McMillan 1960, Bett 1962, Spiera 1966) due to tryptophan pyrrolase enzyme induction (Knox and Mehler 1950). Salicylate displaced non-esterified fatty acids from their binding sites on albumin and these fatty acids could be taken up into cells and broken down. The production of aceto-acetate in this process could play a part in the acidosis which occurs during salicylate toxicity (Dawkins, McArthur and Smith 1970). Rather unusually since it inhibits most aminotransferases, salicylate stimulates L-tryptophan α-oxoglutarate aminotransferase (Gould and Smith 1965).

Tagliamonte, Tagliamonte, Perez-Cruet and Gessa (1971 (b)), and Tagliamonte, Tagliamonte, Perez-Cruet, Stern and Gessa (1971(d)) found that the concentration of tryptophan in the brain reflected 5-hydroxytryptamine synthesis. Conversely Korf, Van Praag and Sebens (1972) and Van Praag, Flentge, Korf, Dols and Schut (1973) found decreased serum tryptophan concentrations, increased brain tryptophan concentrations and unchanged brain 5-hydroxytryptamine concentrations following the administration of probenecid to rats. However Lewander and Sjostrom (1973) recorded increased concentrations of free tryptophan in the plasma of humans, following probenecid administration.

Tryptophan concentrations may also be important in pathological conditions. Curzon, Kantamaneni, Winch, Rojas-Bueno, Murray-Lyon and Williams (1973) posed the question whether increased brain tryptophan could have a role in coma. Hartmann, Chung and Chien (1971) found hypnotic properties for L-tryptophan in man. A dose dependent reduction in sleep latency with little change in sleep architecture was demonstrated in rats, whereas Williams, Lester and Coulter (1968) found slow wave sleep increased and sleep-dream cycle length decreased at similar doses. Wyatt et al. (1970) showed an increase in slow wave sleep

and decreased paradoxical sleep following tryptophan administration, but reduced 5-hydroxytryptamine levels decreased paradoxical sleep. He therefore suggested that tryptophan was acting by a different mechanism, possibly by the kynurenine route.

Lapin and Oxenkrug (1969) and Curzon (1969) suggested that an increased metabolism of tryptophan along the kynurenine pathway, thereby reducing the amount of tryptophan available for conversion to 5-hydroxytryptamine or tryptamine, might participate in the production of the symptoms of mental depression. However, the results of Frazer, Pandey and Mendels (1973) did not support this suggestion, although the former hypothesis (Lapin and Oxenkrug 1969; Curzon 1969), would be in agreement with the most consistent observation in depressed patients, that they have a lower than normal concentration of either 5-hydroxyindoles or 5-hydroxyindole-3-acetic acid in lumbar spinal fluid (Ashcroft, Crawford, Eccleston, Sharman, MacDougall, Stanton and Binns 1966; Dencker, Malm, Roos and Werdinius 1966; Bowers, Heninger and Gerbode 1969; Mendels, Frazer, Fitzgerald, Ramsey and Stokes 1972). A substantial review of the role of indoleamines in affective disorders and schizophrenia has been presented by Himwich (1970).

The regional distribution of tryptophan in the brain of the dog has been plotted by Price and West (1960). Hypothalamus, pons and cerebellum are rich in tryptophan and poor in 5-hydroxytryptamine (approximately a 100 fold difference) whereas cerebral hemispheres and spinal cord show a much smaller ratio (10:1). This difference between tryptophan and 5-hydroxytryptamine concentrations would agree with the view of Morgan and Yndo (1973) that the proportion of brain tryptophan in 5-hydroxytryptamine-containing neurones must be very small.

Tryptophan is taken up by 5-hydroxytryptamine-containing neurones in the brain, and the intrasynaptosomal concentration of L-tryptophan is one factor controlling 5-hydroxytryptamine synthesis (Grahame-Smith and Parfitt 1970). Yuwiler, Wetterberg and Geller (1971) argued against the proposal that the activity of tryptophan peroxidase directly affects the synthesis of brain 5-hydroxytryptamine by diverting tryptophan from the biosynthesis of this monoamine. Tagliamonte, Biggio, Vargiu and Gessa (1973(b)) found indirect evidence indicating that the rate limiting step in the synthesis of 5-hydroxytryptamine is the concentration of tryptophan present and not the activity of tryptophan-5-hydroxylase as suggested by other workers. (Chapter Five).

The synthesis of 5-hydroxytryptamine and 5-hydroxyindole-3-acetic acid from tryptophan has been shown to be partly dependent on substrate levels (Moir and Eccleston 1968; Grahame-Smith 1971; Fernstrom and Wurtman 1972). Schubert (1973), using a transected spinal cord preparation, came to the conclusion that the synthesis of 5-hydroxytryptamine is not directly dependent on neuronal activity or tryptophan levels, but seemed to be inversely correlated to endogenous 5-hydroxytryptamine concentrations, although the possibility that the uptake and transport of tryptophan in 5-hydroxytryptamine neurones may be impaired by spinal transection could not be discounted (Carlsson, Kehr, Lindqvist, Magnusson and Atack 1972). Knott, Joseph and Curzon (1973) postulated that a possible regulatory role of increased brain tryptophan could involve decreased firing of 5-hydroxytryptamine neurones, as shown when tryptophan is injected peripherally (Aghajanian 1972). Moir (1971) illustrated the dangers of regarding total plasma or brain concentrations of tryptophan as indicative of the quality of the substance available for transport or metabolism. "Thus it is naive to relate such concentrations

to the *in vitro* determined K_m values of their metabolising enzymes, or to imply that alteration in total concentration of substrate in whole brain must necessarily alter significantly the rate of subsequent neuronal metabolism (Tagliamonte, Tagliamonte, Perez-Cruet and Gessa 1971(b))".

A diurnal variation in mammalian tryptophan concentrations has been shown (Fernstrom and Wurtman 1971; Wurtman, Rose, Chou and Larin 1968; Morgan and Yndo 1973; Rapoport, Feigin, Bruton and Beisel 1966), and the diurnal variation has been related, in man, to circadian periodicity of tryptophan metabolism (Rapoport and Beisel 1968), and to tyrosine transaminase activity (Wurtman, Shoemaker and Larin 1968; Fuller and Snoddy 1968). Bobillier and Mouret (1971) suggested that the diurnal variation of brain tryptophan concentrations may be partly a reflection of the variation of tryptophan hydroxylation in the brain. In all these instances, both brain and plasma tryptophan concentrations show highest levels following feeding and lowest levels during the period of fasting. However Morgan and Yndo (1973) proposed that the daily rhythm of tryptophan and 5-hydroxytryptamine in mouse brain may be relatively independent of daily variations of food intake or plasma tryptophan content.

Hery, Rouer and Glowinski (1972, 1973) found that the decreased formation of 5-hydroxytryptamine and 5-hydroxyindole-3-acetic acid in hypothalamic slices during the dark period (21.00 h.)is related to changes in the initial accumulation of ^{3}H-tryptophan in tissues, and not related to changes in the rate of conversion of tryptophan to 5-hydroxytryptamine nor was it dependent on plasma tryptophan levels.

In vivo studies of tryptophan uptake into the brain (Guroff and Udenfriend 1962) showed an accumulative process, but brain levels did not reach plasma levels. A marked stereospecificity, with preference for the L-isomer, was demonstrated. Kiely and Sourkes (1972) confirmed the accumulation of tryptophan into rat cerebral cortical slices against its concentration gradient. This accumulation is an energy requiring process (Joanny, Barbosa and Corriol 1968; Barbosa, Joanny and Corriol 1970). Grahame-Smith and Parfitt (1970) have elucidated the nature of tryptophan transport across the synaptosomal membrane, with K_m for the uptake process of $1 \times 10^{-3}M \pm 0.28 \times 10^{-3}M$ and V_{max} 225 ± 39 mμmo/g^{-1}/5 min. in a saturable system, which utilises a stereospecific carrier-mediated process. Karobath, Diaz and Huttunen (1972) found that L-dihydroxyphenyl alnine (L-DOPA), L-3-methoxytyrosine and tryptophan shared an uptake transport system and that these compounds competitively inhibited the uptake of tryptophan into synaptosomes. Catecholamines did not interfere with the uptake of tryptophan into synaptosomes, but both dopamine and noradrenaline inhibited the synthesis of 5-hydroxytryptamine from tryptophan in this system.

A high affinity uptake process has been reported (Knapp and Mandell 1972; Knapp and Mandell 1973; Meek and Neff 1972) with a K_m $1 \times 10^{-5}M$, as opposed to the low affinity process of Grahame-Smith and Parfitt (1970). It has been suggested (Iversen 1970; Shaskan and Snyder 1970) that high affinity amino acid uptake in synaptosomes is indicative of neurotransmitter function, whereas low affinity uptake is related to protein synthesis. The high affinity uptake can be inhibited with cocaine and stimulated with lithium (Knapp and Mandell 1972). Raphe lesions do not affect this process (Kuhar, Roth and Aghajanian 1972) although the uptake of 5-hydroxytryptamine is reduced by such lesions.

The turnover of tryptophan in brain is very rapid (approximately ten minutes) (Tyce, Flock, Owen, Stobie and David 1967), so its availability for conversion to 5-hydroxytryptamine depends on its transport from blood to neurones. At least two processes complicate this transport

1) The high affinity tryptophan uptake system in the nerve endings (Mandell, Knapp and Hsu 1974).
2) Transport into glial cells (Henn and Hamberger 1971).

Bauman, Bourgoin, Benda, Glowinski and Hamon (1974) suggested that variations of plasma free tryptophan could modify the tryptophan carrier activity in glial cells, thence leading to changes in 5-hydroxytryptamine synthesis.

Grahame-Smith (1973) did not find a specific uptake system for tryptophan across the synaptosomal membrane, and suggested that there were"mechanisms" for stabilising the intracellular concentration of tryptophan in the brain.

4.2 METHODS

Male Sprague Dawley rats (120 - 140g) were housed and maintained exactly as described in Chapter Two.

The animals were killed by neck dislocation. Their throats were cut, and blood collected from the wound. Blood was left overnight to clot. The brains were immediately removed as described previously, and homogenised in 6 ml. 0.4M perchloric acid using a glass tube with teflon pestle (0.01 in. clearance), and centrifuged at 3,000g for ten minutes. 4.5 ml. of the supernatant was centrifuged at 25,000g for 20 minutes. 4.0 ml. of the supernatant was taken for assay by the aldehyde condensation method of Denckla and Dewey (1967).

0.1 ml. of a solution containing 0.01M ferric chloride in 85% (w/v) trichloracetic acid and 0.1 ml.18% formaldehyde were added to the sample and boiled together at 100°C for one hour. The reaction mixture was cooled to room temperature and the fluorescence measured at excitation wavelength 360 mμ and emission wavelength 450 mμ (wavelength uncorrected).

For the estimation of serum tryptophan, 0.02 ml. serum was added to 0.2 ml. 1.8% formaldehyde and 1.8 ml. of a solution containing 3×10^{-4}M ferric chloride in 10% (w/v) trichloracetic acid. The reactants were boiled for one hour at 100°C, cooled to room temperature, centrifuged at 25,000g for ten minutes and the fluorescence measured as before.

Serum free tryptophan was determined by dialysing 2.0 ml. serum against 0.5 ml. Krebs-Ringer solution pH 7.4 in a shaking water bath at 37°C for times indicated in the legends to the Figures. 8/32 in. Visking tubing was used as the dialysing membrane. This was boiled twice in 2×10^{-4}M disodium EDTA (ethylenediaminetetracetic acid) and twice in distilled water to remove heavy metal ions, notably copper. The tubes used for the dialysis were flushed with O_2/CO_2 (95:5) prior

to incubation. Tryptophan was assayed as for serum total tryptophan, using 0.02 ml. Krebs-Ringer dialysate.

The twenty-four hour variation of brain and serum free and total tryptophan were determined by measuring the amino acid concentrations at four hourly intervals throughout the twenty-four hour period, commencing at 09.00 h. The twenty-four hour rhythms were compared with the calculated sine curve by means of the Fourier analysis and X^2 test.

The product of the aldehyde-condensation reaction with tryptophan is norharman (Denckla and Dewey 1967). The fluorescence spectrum of the product of the assay was compared with that for pure norharman (Sigma).

Internal tryptophan standards and norharman standards were used for each assay.

4.2.1 RESULTS

The fluorescence spectrum for pure norharman was identical with that of the product being assayed (Figure 22). The results obtained can therefore be considered to be a true measurement of the concentrations of tryptophan in serum and brain.

Serum samples subjected to dialysis at $37^{o}C$ were incubated for 30 mins.,1,2,3,6 and 18 hours. Tryptophan concentrations came rapidly to equilibrium at one hour, and were maintained in this state throughout the eighteen hours of the experiment, although some re-adjustment occurred between two and six hours. A 9% decrease in fluorescence was detected after three hours, but this eventually returned to the equilibrium state. In Figure 23,time of incubation is plotted against fluorescence units, as recorded on the Aminco-Bowman spectrophoto-fluorometer.

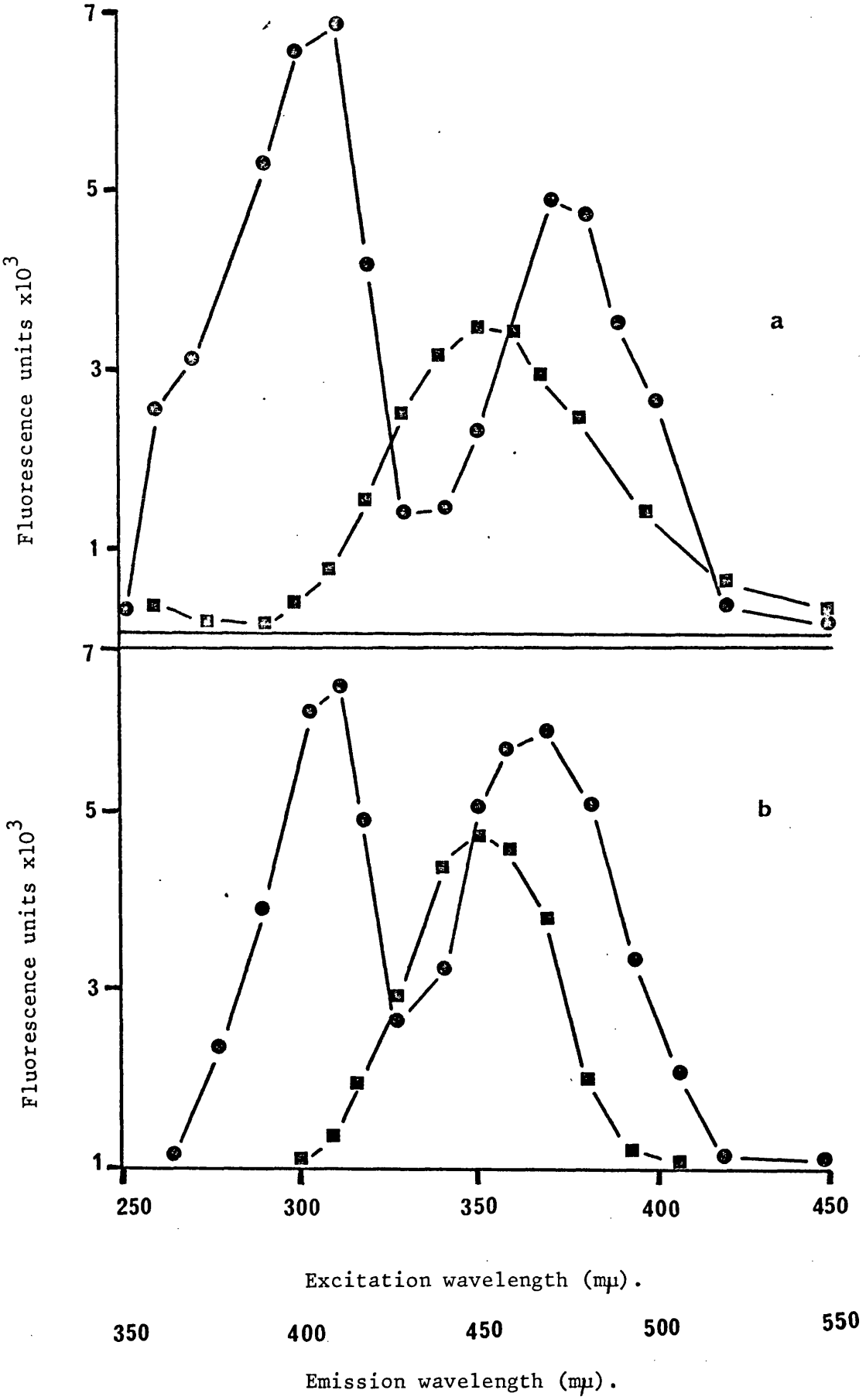


Figure 22. The fluorescence spectrum of the condensed tryptophan product (a) compared with that of pure norharman (b). (■——■) excitation 360mμ, scan emission; (●——●) emission 450mμ, scan excitation.

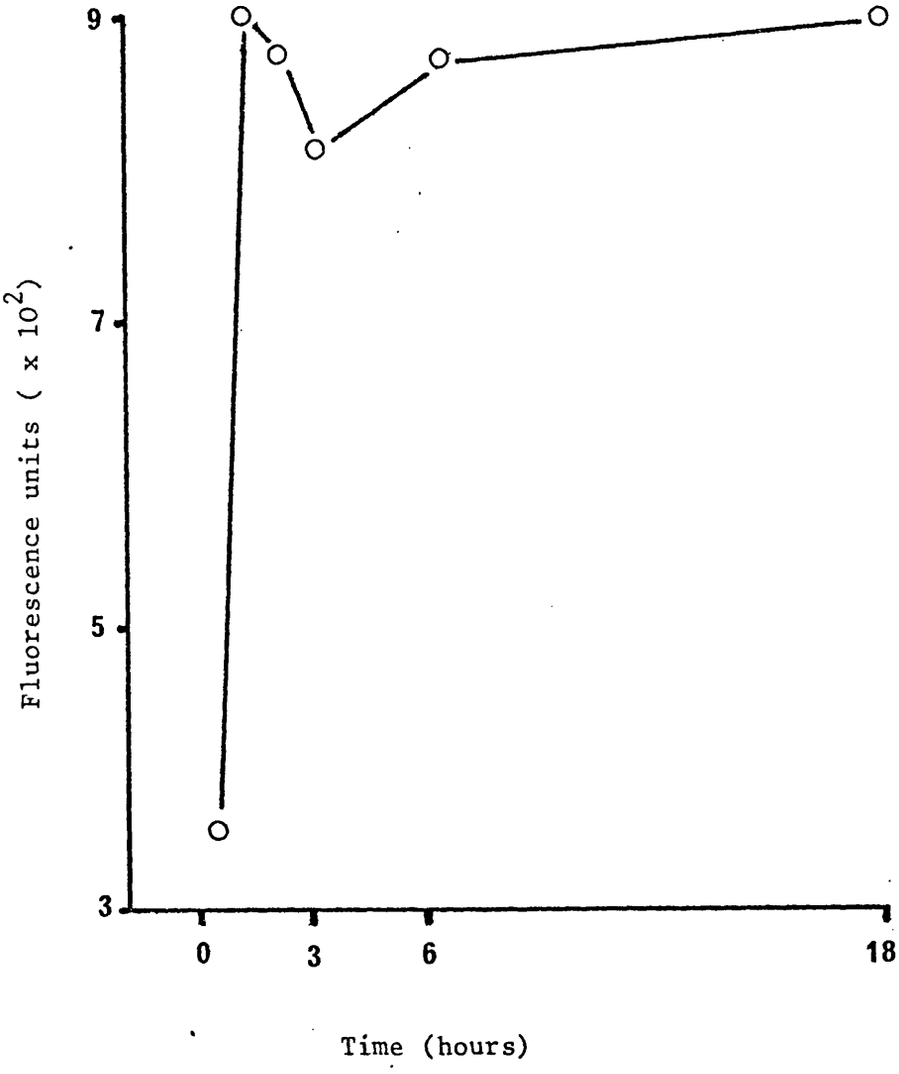


Figure 23. The effect of time on the dialysis of serum tryptophan.

There is a twenty-four hour variation in the concentration of tryptophan in the serum (total and free) and brain (Figure 24, Table 1). The twenty-four hour variation of bound tryptophan in the serum is also shown in Figure 24. These figures were calculated as being the difference between serum total and free tryptophan concentrations. In all instances the highest concentration of tryptophan occurred in the middle of the dark period when the animal is most active and consumes the greatest amount of food. The lowest concentrations occurred in the middle of the light period when the animal is least active. The twenty-four hour variations in the tryptophan concentrations have been compared with their calculated sine curves (Figures 25, 26, 27 for serum free and total tryptophan and brain tryptophan respectively). None of these rhythms was significantly different from their calculated curve when assessed by the X^2 test. Brain tryptophan concentrations fluctuated by 38% throughout the twenty-four hours, serum free tryptophan by 20% and serum total tryptophan by 34%.

In serum and brain, tryptophan concentrations increased rapidly following the onset of darkness (18.00 h.) to maximum concentrations at 01.00 h. Concentrations decreased rapidly after this time in serum, but rather more slowly in the brain. Tryptophan concentrations were at their lowest level by 09.00 h. and remained in this state until 17.00 h. immediately preceding the onset of darkness. Tryptophan concentrations in the brain and in serum appear to increase only immediately following the onset of darkness and maintain a plateau of lower concentrations during the light period and the latter part of the dark period.

28% of serum tryptophan existed in the free form at 01.00 h. and 35% at 13.00 h. The daily mean was 32%. The ratio of free : total tryptophan in the serum does not therefore change significantly throughout the twenty-four hour day length.

Clock hour	TRYPTOPHAN CONCENTRATION (µg/g.brain or µg/ml.serum) ± S.E.M.			
	Brain	Serum 'free'	Serum total	Serum'bound'
01.00	3.59 ± 0.05 (22)	6.41 ± 0.22 (15)	22.89 ± 0.73 (15)	16.48
05.00	3.28 ± 0.05 (21)	5.34 ± 0.72 (16)	15.98 ± 1.10 (16)	10.64
09.00	2.23 ± 0.04 (20)	5.19 ± 0.18 (14)	17.10 ± 1.00 (14)	11.91
13.00	2.36 ± 0.08 (22)	5.36 ± 0.24 (21)	15.04 ± 0.46 (21)	9.68
17.00	2.38 ± 0.06 (16)	5.16 ± 0.37 (15)	17.38 ± 1.10 (15)	12.22
21.00	3.16 ± 0.05 (15)	6.14 ± 0.21 (14)	21.54 ± 1.00 (14)	15.40
13.00	2.35 ± 0.05 (8)	5.21 ± 0.21 (8)	15.10 ± 0.77 (8)	9.89

The number of animals used at each clock hour is shown in parentheses.

Table 1. The concentrations of tryptophan in the brain and serum of the rat.

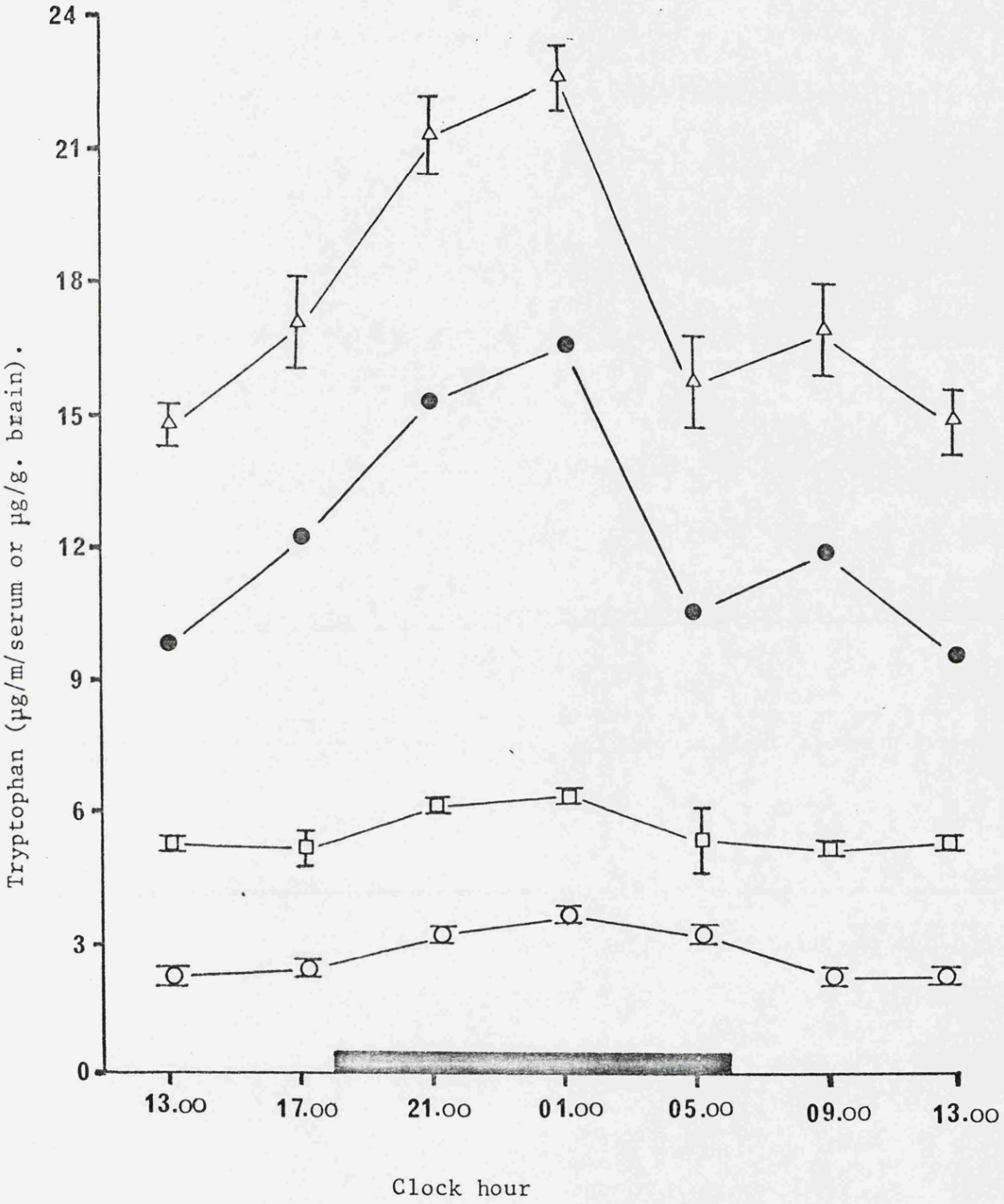


Figure 24. The twenty-four hour variation of tryptophan concentrations in the rat brain (O —— O) and serum 'free' (□ —— □) , 'bound' (● —— ●) and total (△ —— △). The solid black bar represents the period of darkness.

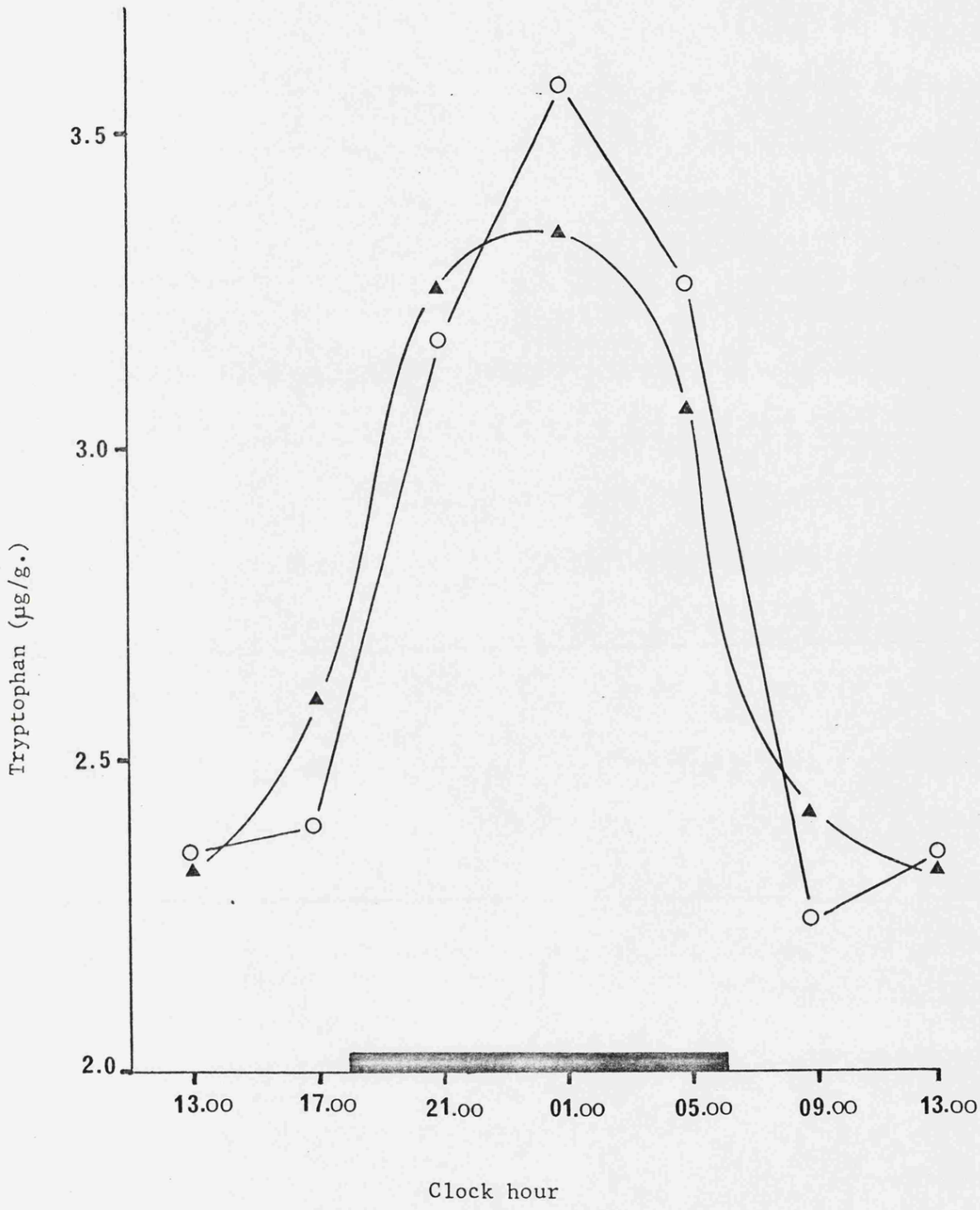


Figure 25. The twenty-four hour variation of tryptophan concentrations in the rat brain (O——O) compared with the calculated sine curve (▲——▲).

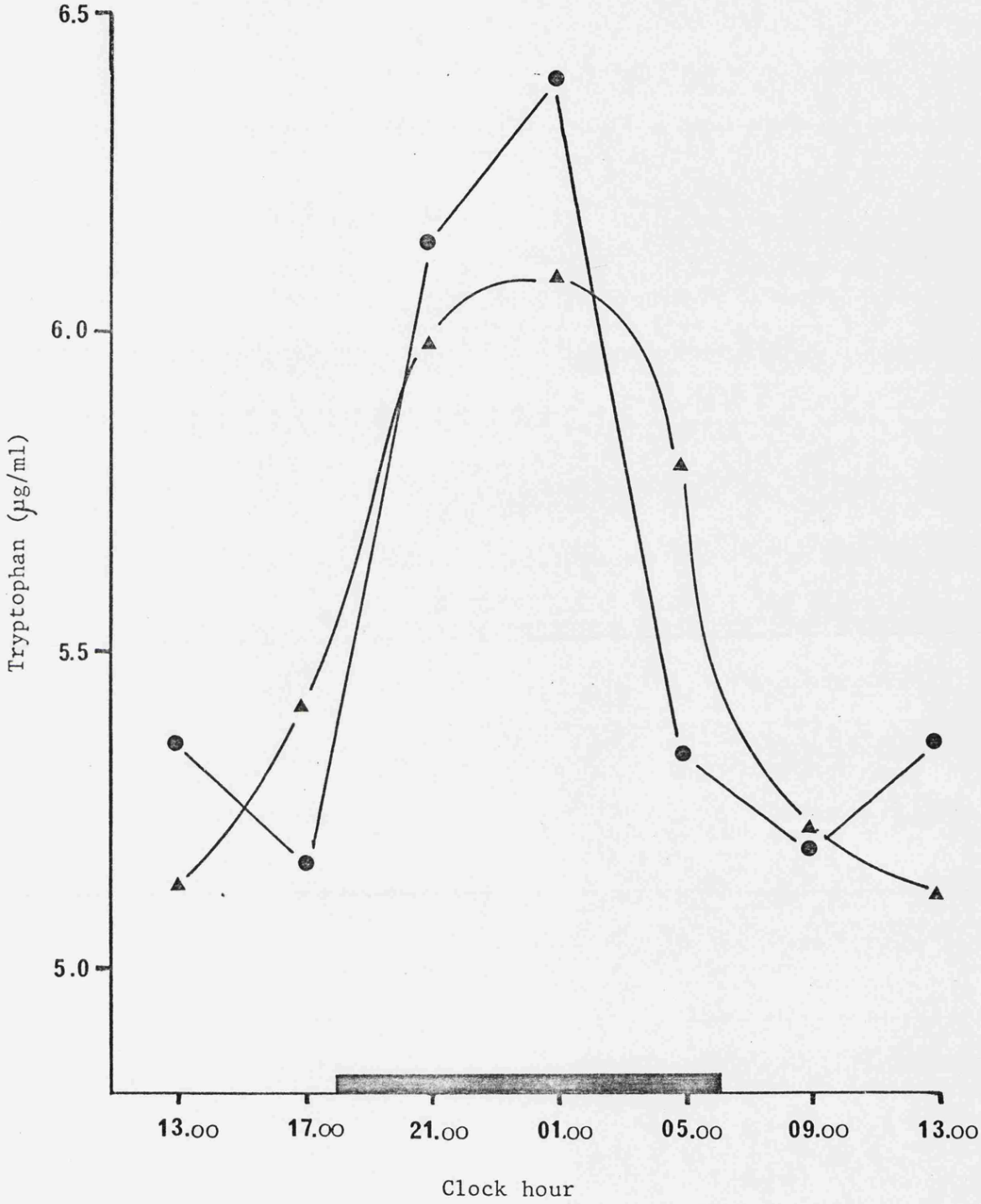


Figure 26. The twenty-four hour variation of serum free tryptophan (●——●) compared with the calculated sine curve (▲——▲).

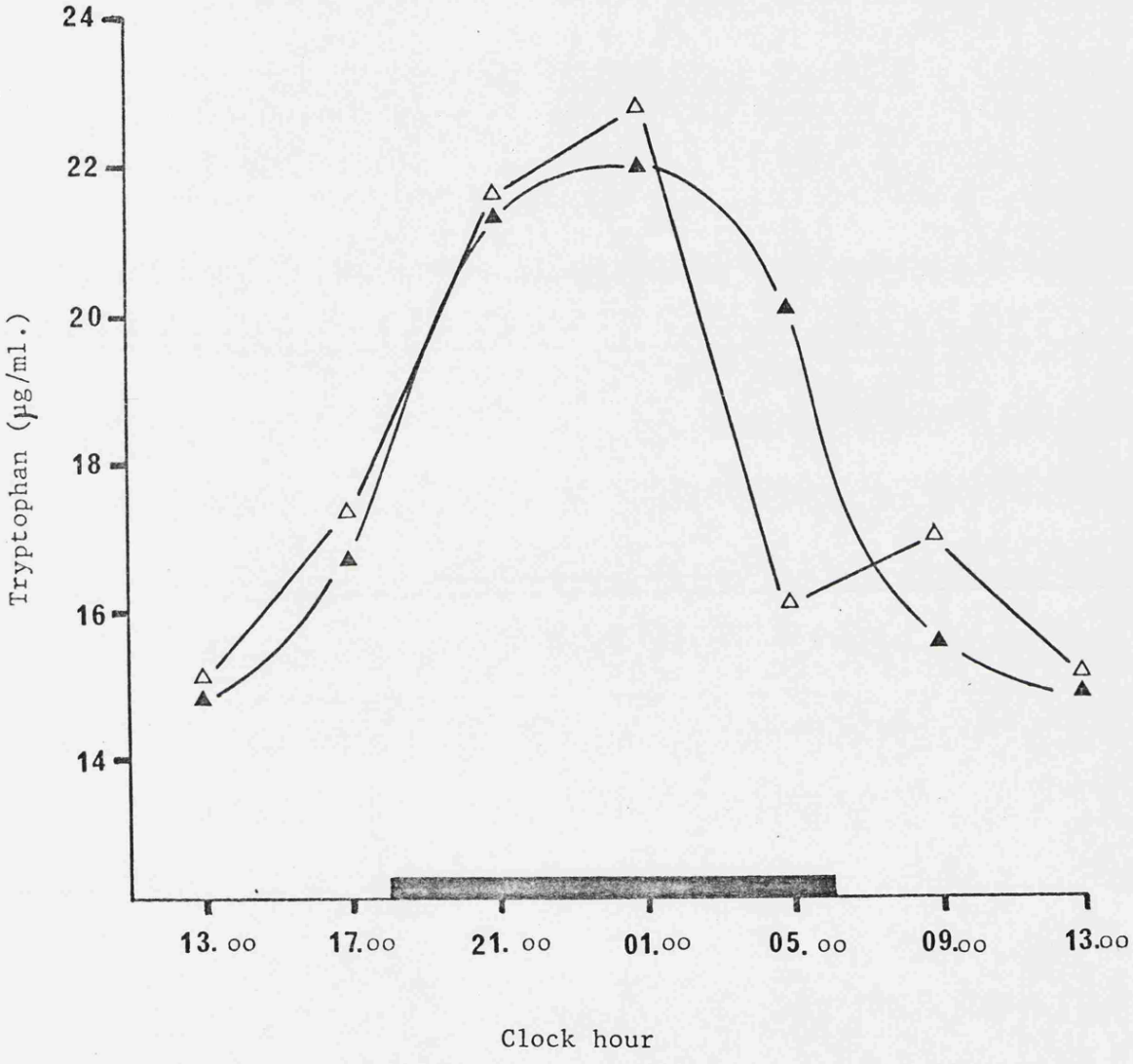


Figure 27. The twenty-four hour variation of serum total tryptophan concentrations (△——△) compared with the calculated sine curve (▲——▲).

4.3 The Uptake of ^{14}C-Tryptophan into nerve endings

METHODS

The uptake of ^{14}C-tryptophan into homogenates of the septal region of rat brain, was determined exactly as described for the uptake of ^{14}C-5-hydroxytryptamine in septal regions (Chapter Three), with the exception that DL-^{14}C-tryptophan 57 mCi/m mole was used as the substrate. The effects of incubation time, and the Michaelis constant for the uptake of tryptophan were determined.

In addition the uptake of DL-^{14}C-tryptophan into a synaptosomal fraction was determined as follows.

Male Sprague Dawley rats (120 - 140g) were maintained as described previously for ten days before experimentation. The rats were killed by decapitation and the brains were rapidly removed, weighed and homogenised in 0.32M sucrose (10 ml/g.) and centrifuged at 1,000g at 4^{o}C for ten minutes. The supernatant was removed and centrifuged at 17,000g at 4^{o}C for one hour. The supernatant was discarded, and the pellet resuspended in 20 mls. Krebs-Ringer solution pH 7.4.

This fraction was placed on a discontinuous sucrose gradient of composition 0.32M, 0.8M, 1.2M sucrose and centrifuged at 100,000g for one hour at 0^{o}C in a Beckman ultracentrifuge using a S.W.25.2 swing out rotor. The synaptosome fraction was collected at the interface of the 0.8M and 1.2M sucrose solutions. 5 mls. of the synaptosome fraction were adjusted to 0.4M by slowly adding an equal volume of water. To 1.0 ml. of this, 8 ml. of Krebs-Ringer solution was added and this mixture was preincubated at 37^{o}C for five minutes. 1.0 ml. DL-^{14}C-tryptophan 2 x 10^{-6}M made up in Krebs-Ringer solution pH 7.4 was added, and the effect of incubation time on the uptake

process was determined. The final concentration of DL-^{14}C-tryptophan was 2 x 10^{-7}M.

The uptake process, by these two methods, was compared at two different times of the day, 01.00 h. and 13.00 h. to determine any variation in the process with clock hour.

The concentrations of isotope used, and the incubation times are presented in the legends to the Figures. All results given are the means of three experiments.

4.3.1 RESULTS

The uptake of DL-^{14}C-tryptophan(2 x 10^{-7}M)into a homogenate of the septal region of rat brain increases with time up to a maximum after eight minutes, in samples taken at 01.00 h. and 13.00 h. (Figure 28). Samples taken at both clock hours have equal initial rates of uptake, and the uptake of DL-^{14}C-tryptophan with time, does not therefore display a twenty-four hour variation at this concentration (2 x 10^{-7}M). The concentration of amino acid decreased following attainment of maximum uptake. The reason for this is rather obscure, but two possibilities may be suggested. First tryptophan may be taken up, metabolised and released, and therefore becomes unavailable for re-uptake with the result that there is insufficient tryptophan available to replenish that which is released. Secondly the stability of the nerve endings in the homogenate may decrease with time. Rupture of nerve endings would limit the uptake sites available, and if this was a continuous process, it might be expected that the amount of amino acid taken up would decrease. The former possibility does not seem likely, since even at higher concentrations (10^{-4}M), when one would expect excess substrate to be

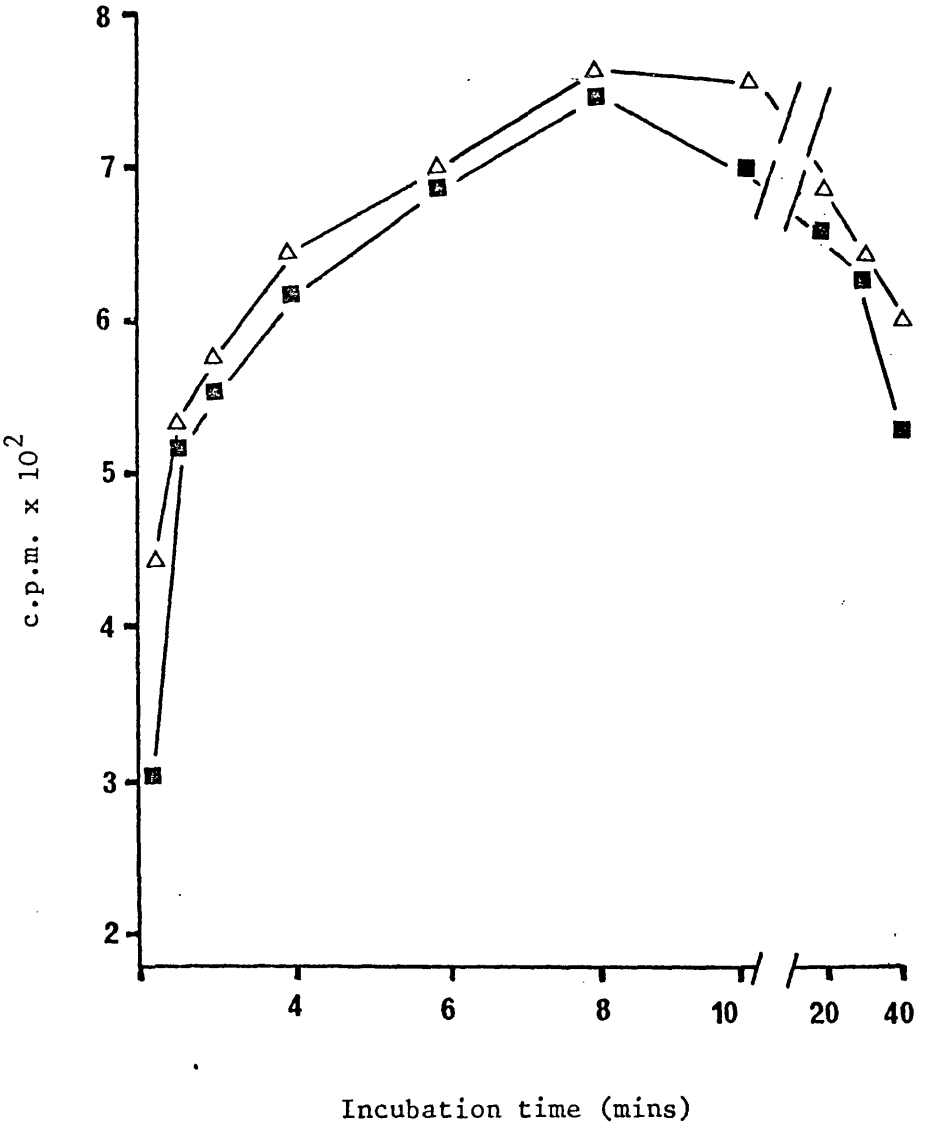


Figure 28. The effects of incubation time on the uptake of ^{14}C-tryptophan (2 x 10^{-7}M) into homogenates of the septal region of rat brains at 01.00 h. (△——△) and 13.00 h (■——■) at 37°C and pH 7.4.

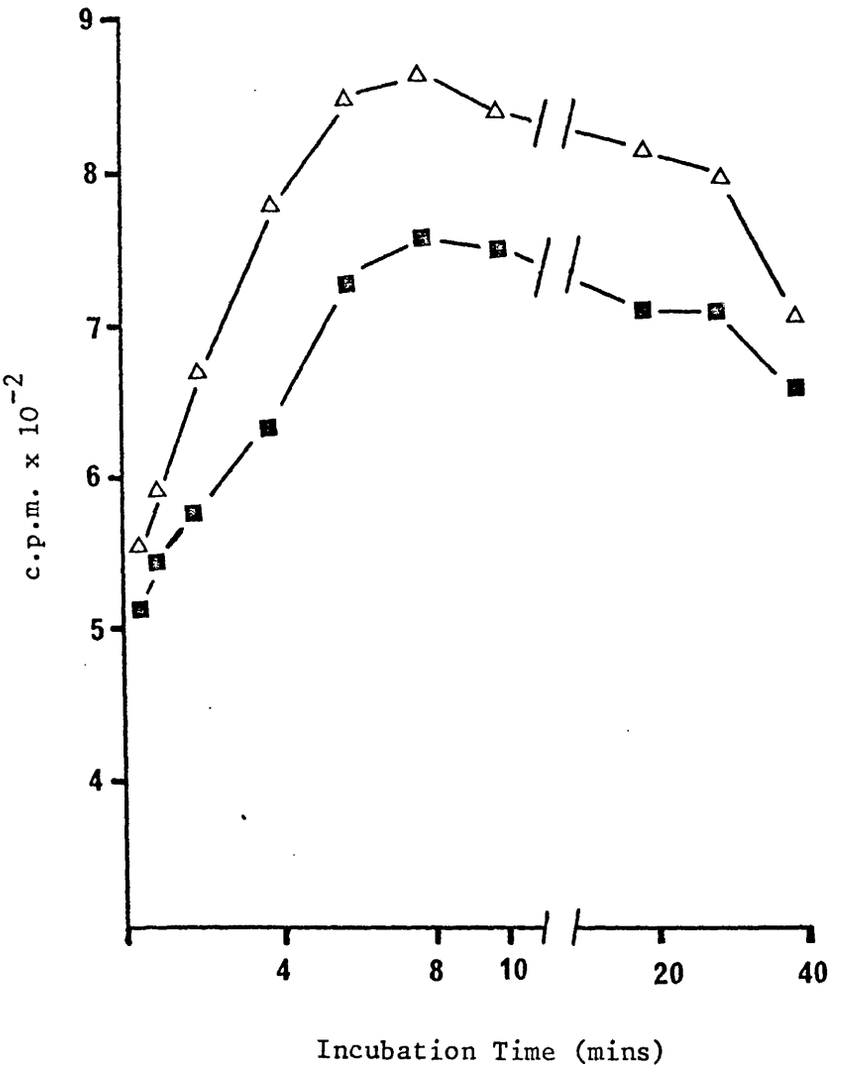


Figure 29. The effects of incubation time on the uptake of ^{14}C-tryptophan (2 x 10^{-7}M) into a synaptosomal preparation from rat brain at 01.00 h (■——■) and 13.00 h (△——△) at 37°C and pH 7.4.

present, there is still a decrease in uptake at incubation greater than twenty minutes. The second possibility, if true, would mean that the breakdown of the homogenate must occur throughout the experiment and the results are not a true estimate of the uptake capabilities of the homogenate. However, these results are only being used to compare the rates of uptake at two clock hours, and the results remain valid unless one accepts the possibility of a twenty-four hour rhythm in the rate of breakdown of the homogenate. It was interesting to note that in each of the three experiments, of which the mean is shown in Figure 28, the uptake curve for samples taken at 13.00 h. was always higher than that for 01.00 h, although there is no statistically significant difference between them.

The uptake of DL-^{14}C-tryptophan into the prepared synaptosomal fraction was similar to that for the crude homogenate of the septal region. Maximum uptake occurred after eight minutes (Figure 29). The rate of uptake for samples taken at 01.00 h. was similar in the two systems. The rate of uptake for samples taken at 13.00 h. was 30% greater in the synaptosomal fraction than in the crude homogenate and 33% greater than in the synaptosomal fraction taken at 01.00 h. There was a decline in tryptophan uptake with time after maximum levels of uptake had been reached.

The Michaelis constant (K_m) was plotted for samples taken at 01.00 h. and 13.00 h. (Figures 30, 31). The K_m for the uptake of DL-^{14}C-tryptophan into a homogenate of septal region of rat brain was 6.2×10^{-5}M for samples taken at 13.00 h. and 3.0×10^{-5}M for samples taken at 01.00 h. There is no difference in the K_m values at these clock hours, and consequently it must be concluded that there is no twenty-four hour variation of the Michaelis constant for DL-^{14}C-tryptophan.

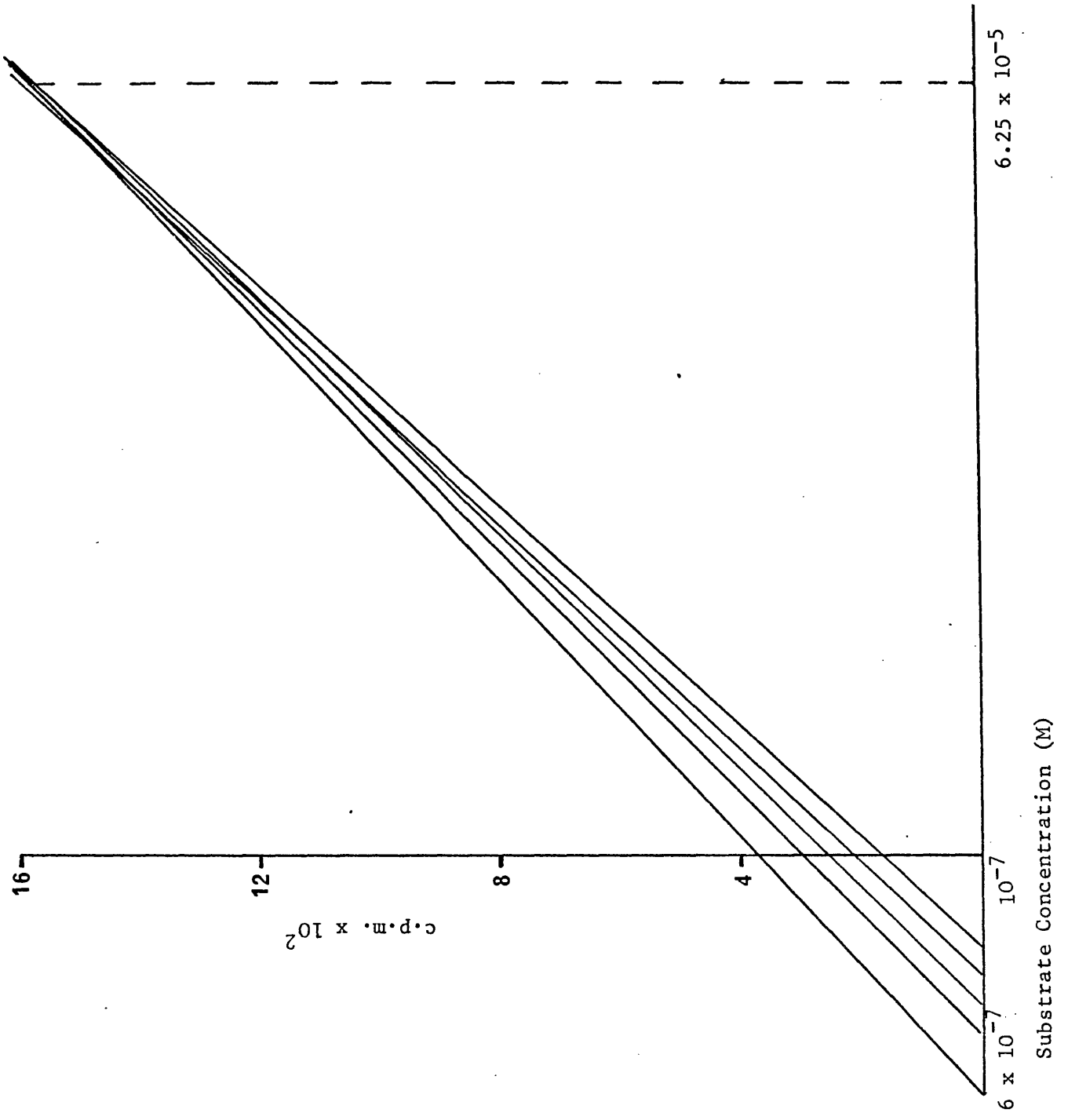


Figure 30. Direct linear plot to determine the Michaelis constant for the uptake of ^{14}C-tryptophan into a septal homogenate at 13.00 h., 37°C and pH 7.4.

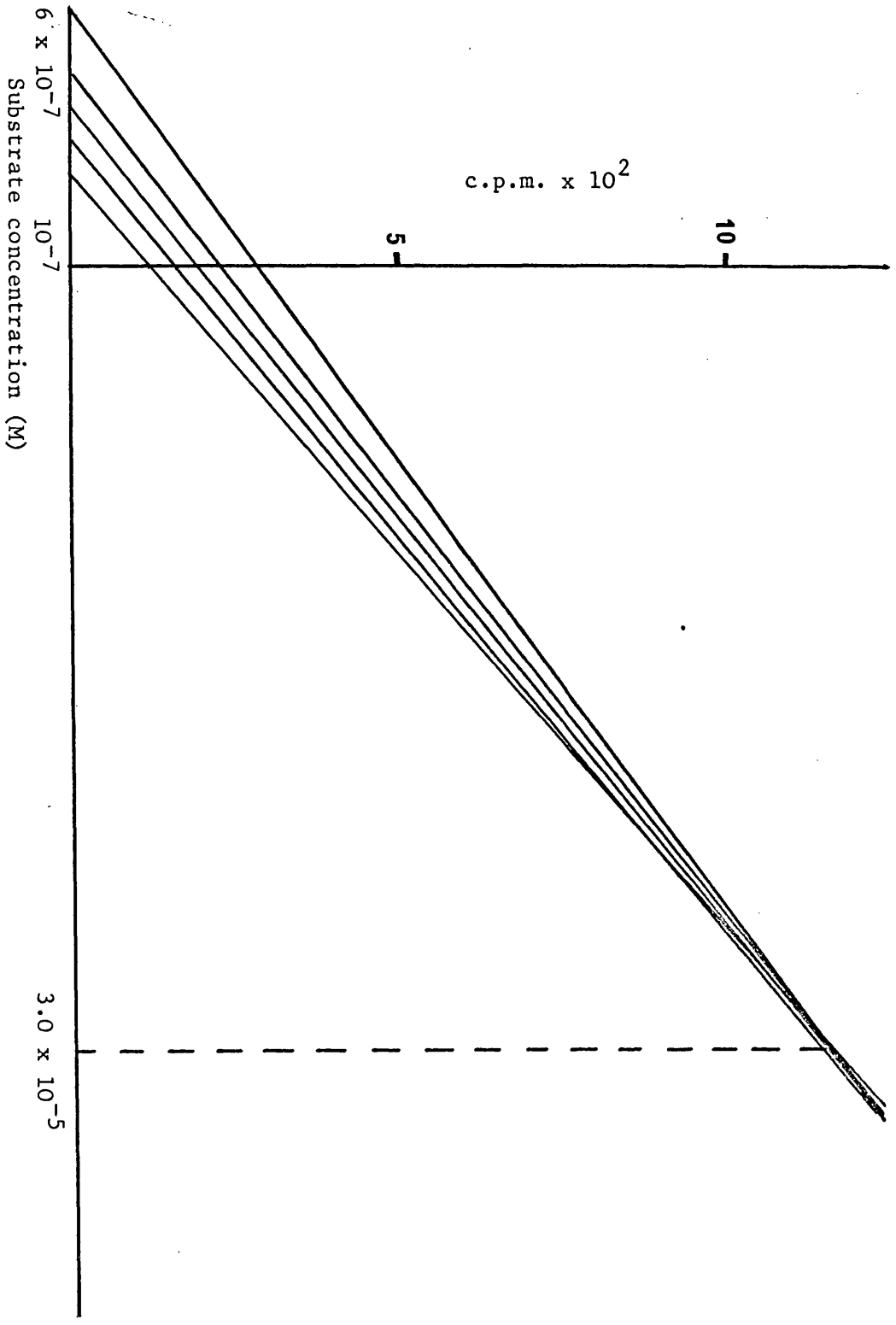


Figure 31. Direct linear plot to determine the Michaelis constant for the uptake of ^{14}C-tryptophan into a septal homogenate at 01.00 h., 37°C, pH 7.4.

As presented previously (Chapter Three) V_{max} has been calculated (Figures 30 and 31) without taking the different endogenous concentrations of tryptophan into account, and V_{max} was approximately 26% lower at 01.00 h. than at 13.00 h. under these conditions. However, when V_{max} was recalculated to take the different endogenous concentrations of tryptophan into account, the level at 01.00 h, was approximately 8% higher than at 13.00 h. There was no statistically significant difference between the values of V_{max} measured at 01.00 h. and 13.00 h, and it must therefore be concluded that V_{max} does not display a twenty-four hour variation.

4.4 DISCUSSION

The twenty-four hour variations in serum free and total tryptophan and brain tryptophan are similar, with highest concentrations in the dark period and lowest concentrations in the light period, thus agreeing with results shown by other workers (Fernstrom and Wurtman 1971). Similarly the characteristics of the high affinity uptake process (K_m 6.2 x 10^{-5}M at 13.00 h; 3.0 x 10^{-5}M at 01.00 h) agrees with the findings of other workers (Knapp and Mandell 1972).

The most striking anomaly when these results are compared with the twenty-four hour variation of 5-hydroxytryptamine in Chapter Three is the twelve hour difference between the highest concentrations observed for tryptophan (01.00h)and for 5-hydroxytryptamine (13.00 h). It is therefore apparent that the twenty-four hour variation of 5-hydroxytryptamine is not merely a consequence of increased tryptophan concentration. Hery, Rouer and Glowinski (1972) related the increase in 5-hydroxytryptamine to the increase in initial rate of accumulation

of tryptophan. I have little evidence to support this; although a more rapid rate of uptake of tryptophan was seen at 13.00 h. in synaptosomes. This was not reflected in the V_{max} when the Michaelis constants were plotted. Indeed the V_{max} at both times of daywere almost identical. From these results one might suggest that the higher rate of accumulation at 13.00 h. in synaptosomes might play a part in the establishment of the twenty-four hour variation of 5-hydroxytryptamine although it would be necessary to establish a similar variation in V_{max} to confirm this.

If the other implications of the twenty-four hour variation of tryptophan in the brain are considered, it is necessary to find a reason for this variation, since it is obviously not to control 5-hydroxytryptamine concentrations. It may be that the concentration of amino acid in the brain simply reflects the concentration in the serum, either free or bound, since the ratio between these did not change throughout the day. Other authors have suggested that it is the serum free tryptophan that regulates the concentration of the amino acid in the brain (Knott and Curzon 1972), but whether the serum free tryptophan actually regulates brain tryptophan concentrations or whether brain tryptophan concentrations are merely a reflection of peripheral metabolism is debateable.

It appears from this work that the amount of tryptophan required for the synthesis of 5-hydroxytryptamine cannot be determined from the measurement of concentrations of tryptophan in the brain, particularly by the methods employed, which have only measured gross changes in tryptophan concentration.

If it is true that the synthesis of 5-hydroxytryptamine is independent of total brain tryptophan concentration, and this seems likely since the highest concentration of 5-hydroxytryptamine coincides with the lowest concentration of tryptophan, then only a small proportion of tryptophan can be involved in the synthesis of the amine, and the concentrations involved must be very carefully controlled. What controls the availability of tryptophan for the conversion to 5-hydroxytryptamine? The utilisation of tryptophan in protein biosynthesis is an obvious alternative pathway which may limit the availability of tryptophan for the synthesis of 5-hydroxytryptamine but the complexities involved in the suggestion that protein biosynthesis is itself so rigidly controlled make this idea unattractive, and certainly no proof is available. It is more tempting to suggest that the neuronal uptake of tryptophan is the controlling factor, and indeed Hery, Rouer and Glowinski (1972) came to this conclusion by indirect means. However I have found little evidence to support this, and if the initial accumulation of tryptophan were the regulating factor of the twenty-four hour rhythm of 5-hydroxytryptamine concentrations, one might expect the kinetic studies undertaken in this Chapter to vary with clock hour to a degree comparable with the 40% fluctuation of 5-hydroxytryptamine concentrations recorded in the rat brain (Chapter Three).

CHAPTER FIVE

The Effects of Tryptophan-5-hydroxylase activity on the Twenty-four hour variation of 5-hydroxytryptamine.

5.1 INTRODUCTION

Freedland, Wadzinski and Waisman (1961(a) and (b)) found an enzyme able to convert tryptophan to 5-hydroxytryptophan in the liver. Attempts were therefore made to identify the enzyme, tryptophan-5-hydroxylase (E.C. 1.99.1.4.) in the brain.

In this introduction I shall provide evidence to demonstrate the existence and distribution of tryptophan-5-hydroxylase in the CNS, and also the characteristics and regulating factors of the enzyme which play an important part in 5-hydroxytryptamine synthesis. In addition the role of inhibitors of the enzyme will be discussed with respect to the synthesis of the amine.

Initial attempts to demonstrate the presence of tryptophan-5-hydroxylase in the CNS proved unsuccessful (Renson, Weissbach and Udenfriend 1962), and it was not until 1963 that Gal and his co-workers established the presence of the enzyme in vivo (Gal, Poczik and Marshall 1963). The existence of the enzyme in the CNS has since been shown by many workers (Gal, Morgan, Chatterjee and Marshall 1964; Weber and Horita 1965; Consolo, Garattini, Ghielmetti, Marselli and Valzelli 1965).

Gal, Morgan, Chatterjee and Marshall (1964) were unable to demonstrate sufficient enzymic activity to account for all the 5-hydroxytryptamine in the brain, and they therefore concluded that an extra-cerebral source of 5-hydroxytryptophan must complement that which is produced in the brain. However, Bertaccini (1960) did not find a decrease in brain 5-hydroxytryptamine concentrations following removal of the gastro-intestinal tract, as might have been expected. More recent evidence (Garattini and Valzelli 1965; and Grahame-Smith 1967) has indicated the rate of 5-hydroxytryptamine synthesis to be 1 $\mu g/g^{-1}/h^{-1}$ and 0.8 $\mu g/g^{-1}/h^{-1}$ respectively, and both Grahame-Smith (1967) and Jequier, Lovenberg and Sjoerdsma (1967) concluded that

there may indeed be sufficient enzyme to account for all the 5-hydroxytryptamine in the brain.

The decrease of brain 5-hydroxytryptamine concentrations following electrolytic destruction of the midbrain raphe nuclei (Kuhar, Roth and Aghajanian 1971) without a concomitant fall in noradrenaline, dopamine and tryptophan concentrations, indicates that the tryptophan-5-hydroxylase is predominantly localised in 5-hydroxytryptamine-containing neurones.

The regional distribution of the enzyme has been studied in guinea pig brain (Ichiyama, Nakamura, Nishizuka and Hayaishi 1968), cat brain (Peters, McGeer and McGeer 1968) and in rat brain (Deguchi and Barchas 1972). Although there is some species variation, the highest activity has been generally found in the hypothalamus, midbrain, pons and medulla. The activity of tryptophan-5-hydroxylase parallels 5-hydroxytryptamine concentrations (Deguchi and Barchas 1972; Baumgarten, Victor and Lovenberg 1973), 5-hydroxytryptophan decarboxylase activity (Grahame-Smith 1967) and synaptosomal uptake of 5-hydroxytryptamine (Kuhar, Aghajanian and Roth 1972), suggesting perhaps that 5-hydroxytryptamine is synthesised and stored (and therefore found) at the same site.

Tryptophan-5-hydroxylase was thought to be a cytoplasmic enzyme (Lovenberg, Jequier and Sjoerdsma 1968). However Knapp and Mandell (1972(a)) established the presence of both soluble (68%) and particulate (32%) forms of the enzyme in the brain. The soluble fraction was associated with brain regions containing cellbodies e.g. pons, medulla and ventral midbrain, and the particulate fraction with regions containing nerve endings e.g. septum (Grahame-Smith 1967). The activity of the enzyme in the particulate fraction is regulated

by the uptake of substrate, while the activity in the soluble fraction is regulated by the activity of the enzyme itself (Knapp and Mandell 1972(b)).

Meek and Neff (1972) found that tryptophan-5-hydroxylase was apparently synthesised in cell bodies and was carried to the nerve endings by axoplasmic flow at the slow rate of 5 - 7 mm per day. The apparent half life of the enzyme was 2.5 days. Clineschmidt, Pierce and Lovenberg (1971) recorded an 81% reduction in tryptophan-5-hydroxylase activity and 86% reduction of 5-hydroxytryptamine concentrations in the spinal cord following spinal transection, and showed that the enzyme was specifically associated with 5-hydroxytryptamine-containing neurones. There was a relatively constant ratio of enzyme to amine from cell bodies to terminal axons at least in the bulbo-spinal 5-hydroxytryptamine containing neurones.

Study of the maturation of tryptophan-5-hydroxylase in the brain (Deguchi and Barchas 1972) indicated that adult levels were reached after thirty days, and that the maturation of the enzyme paralleled that of 5-hydroxytryptamine, thus suggesting a rate limiting function for the enzyme. Schmidt and Sanders-Bush (1971) found a four-to-five fold increase in tryptophan-5-hydroxylase activity in rats between seven and twenty-eight days post partum. This increase occurs at the same time as dendrite proliferation and increased intraneuronal interactions (Eayrs and Goodhead 1959), the appearance of synaptic junctions (Aghajanian and Bloom 1967(b)), the development of many enzyme systems (Pitts and Quick 1967; Schmidt, Palmer, Dettborn and Robison 1970) and the assumption of adult patterns of electrical activity in the brain (Crain 1952). Bennett and Giarman (1965) and Schmidt and Sanders-Bush (1971) found that the intraperitoneal

injection of tryptophan into the developing animal did not increase 5-hydroxytryptamine concentrations, but 5-hydroxytryptophan injection did, again suggesting a rate limiting function for tryptophan-5-hydroxylase (Deguchi and Barchas 1972). Substrate concentration was not a limiting factor in these experiments.

For many years the dispute as to whether tryptophan-5-hydroxylase or other factors e.g. tryptophan availability (Jequier, Lovenberg and Sjoerdsma 1967), oxygen concentration (Green and Sawyer 1966; Friedman, Kappelman and Kaufman 1972) or a pteridine co-factor (Koe and Weissman 1966) are the controlling influence on 5-hydroxytryptamine synthesis, has been contested. Lovenberg, Jequier and Sjoerdsma (1967) thought that the activity of tryptophan-5-hydroxylase in a neurone, probably determines the rate of synthesis of 5-hydroxytryptamine. Meek and Neff (1972) found that acute changes in the turnover of the amine in nerve endings were not likely to be caused by corresponding changes in synthesis and transport of new tryptophan-5-hydroxylase, but transient changes were probably modulated by other mechanisms e.g. allosteric reactions or availability of substrates or co-factors. In a recent report, Renson (1973(b)) concluded that "The regulation of 5-hydroxytryptamine synthesis cannot be accomplished by rapid changes of enzyme levels".

Ashcroft, Eccleston and Crawford (1965); Green and Sawyer (1966) and Moir and Eccleston (1968) found tryptophan-5-hydroxylase to be rate limiting <u>in vivo</u> and <u>in vitro</u>, but Airaksinen, Giacalone and Valzelli (1968) thought that the enzyme may be partially dependent on the availability of its substrate. Azmitia, Algeri and Costa (1970) suggested that brain stem concentrations of tryptophan-5-hydroxylase exceed the amount required to maintain the steady state concentration of 5-hydroxytryptamine, possibly because the brain stem contains

mainly cell bodies, where the enzyme is presumably synthesised. Clineschmidt, Pierce, and Lovenberg (1971) proposed that the concentration of enzyme determines the concentration of amine in a given area of the brain, but they could not reconcile this with the increase in enzyme without an accompanying rise in amine in the brain stem, following chronic spinal transection. Grahame-Smith and Parfitt (1970) were under the impression that if tryptophan-5-hydroxylase were the controlling factor in the synthesis of 5-hydroxytryptamine in the brain, and in the amount available for functional activity, it would seem to be excessively vulnerable to amino acid inbalance in the plasma. Sheard and Aghajanian (1968); Kuhar, Roth and Aghajanian (1971); Shields and Eccleston (1972) and Carlsson, Lindqvist, Magnusson and Atack (1973) found that nerve impulse flow influenced tryptophan-5-hydroxylase activity to some extent. Shields and Eccleston (1972) concluded that the rise in synthesis of 5-hydroxytryptamine, as a result of electrical stimulation in the region of the midbrain raphe nucleus, was not due to the induction of the enzyme, but was more likely due to an increase in the activity of existing enzyme. Millard, Costa and Gal (1972) found 5-hydroxytryptamine synthesis to be attuned to circulating corticosteroid via tryptophan-5-hydroxylase, and Azmitia and McEwen (1969) found a 75% decrease in the activity of the enzyme following adrenalectomy.

Tryptophan-5-hydroxylase displays an affinity constant for tryptophan of 5×10^{-4}M, which when compared to brain concentration of the substrate of approximately 1×10^{-5}M, suggests that the enzyme is unsaturated with regard to its substrate (Grahame-Smith 1964; Lovenberg, Jequier and Sjoerdsma 1968; Ichiyama, Nakamura, Nishizuka and Hayaishi 1968). Saturation of tryptophan-5-hydroxylase <u>in vivo</u> by its substrate seems to be accomplished at 0.09×10^{-3}M, and

tryptophan concentrations greater than 0.4×10^{-3}M may give substrate inhibition of tryptophan-5-hydroxylase (Renson 1973(b)). However in experiments using tetrahydropterin (BH_4) or 6-methyl-5,6,7,8 tetrahydropteridine ($6MPH_4$) instead of the more commonly used co-factor 2-amino-4 hydroxy-6,7, dimethyl 5,6,7,8 tetrahydropteridine ($DMPH_4$) (Friedman, Kappelman and Kaufman 1972), the affinity of tryptophan-5-hydroxylase for tryptophan may be within the range of physiological concentrations of tryptophan and therefore the enzyme may not be unsaturated in situ.

The possibility that tryptophan-5-hydroxylase activity may be modified by a negative feedback system, controlled by 5-hydroxytryptamine concentrations, has been another subject of great debate. Macon, Sokoloff and Glowinski (1971) and Hamon Bourgoin and Glowinski (1973) found good evidence for a negative feedback system for the enzyme but Lin, Neff, Ngai and Costa (1969); Millard and Gal (1971) and Millard, Costa and Gal (1972) found no evidence for such an action. Lovenberg, Jequier and Sjoerdsma (1968) and Knapp and Mandell (1973) showed that 5-hydroxytryptamine inhibits the enzyme only in high concentrations (1×10^{-3}M) and in a non-competitive way. The high concentrations of 5-hydroxytryptamine required *in vitro* apparently rule out the possibility of a negative feedback system, but experiments *in vivo* would suggest that a decrease in amine synthesis can be achieved by increasing extra-vesicular intra-neuronal 5-hydroxytryptamine and vice versa. In addition, a moderating influence on drug effects has been shown to occur via tryptophan-5-hydroxylase (Knapp and Mandell 1972(a), 1973) indicating some form of feedback control, although it cannot be ruled out that this will occur indirectly via other systems. Mandell, Knapp and Hsu (1974) found

dopamine to inhibit tryptophan-5-hydroxylase at concentrations similar to those found in the brain.

The nature of tryptophan-5-hydroxylase co-factors has been extensively researched, although the components have not been isolated. Nakamura, Ichiyama and Hayaishi (1965) found that solubilised fore-brain enzyme was stimulated by a pteridine co-factor. Gal, Armstrong and Ginsberg (1966) located the enzyme in mitochondria and showed a requirement for a pteridine type or reduced biopterin co-factor or for NADPH. Green and Sawyer (1966); Davis and Carlsson (1973) showed an oxygen requirement for tryptophan-5- hydroxylase. Grahame-Smith (1967) found no increase in enzyme activity, in intact synaptosomes, following the addition of $DMPH_4$, but rupture of the synaptosomes, or partial purification of the enzyme, revealed that tryptophan-5-hydroxylase was dependent upon $DMPH_4$ for its activity. Friedman, Kappelman and Kaufman (1972), using a partially purified enzyme system, showed an absolute requirement for tetrahydropterin. The enzyme was stimulated by reduced pyridine nucleotide, dihydropteridine reductase and catalase. Fe^{2+} stimulated the enzyme in the absence of catalase, suggesting that Fe^{2+} or catalase protects a sensitive component of the tryptophan hydroxylation system from inactivation by peroxide. Dihydropteridine reductase has been shown to be present in large excess (Craine, Hall and Kaufman 1972).

Inhibition of tryptophan-5-hydroxylase decreases the synthesis and therefore the concentration of 5-hydroxytryptamine in the CNS. Drugs which inhibit this enzyme are useful pharmacological tools in that they provide another means of determining the role of 5-hydroxytryptamine in the CNS. Of these drugs p-chloroamphetamine and p-chlorophenylalanine (p-CPA) have been most widely investigated. p-Chloroamphetamine decreases 5-hydroxytryptamine and 5-hydroxyindole-3-acetic acid concentrations in

the brain by inhibiting tryptophan-5-hydroxylase activity (Sanders-Bush, Bushing and Sulser 1972). The drug is long acting, up to four months, and may therefore be used to study the long term effects of 5-hydroxytryptamine depletion.

Koe and Weissman (1966) reported that p-CPA depleted 5-hydroxytryptamine stores in the brain to very low levels, although brain noradrenaline and dopamine concentrations were only slightly decreased. p-CPA inhibited tryptophan and phenylalanine hydroxylation in the liver, but the drug was more specific to tryptophan-5-hydroxylase in the brain. The slow onset of action of p-CPA may be due to its conversion to an active metabolite, p-chlorophenylpyruvic acid, which has virtually the same effect as p-CPA. Jequier, Lovenberg and Sjoerdsma (1967) found that p-CPA was a competitive inhibitor of tryptophan-5-hydroxylase *in vitro*, but caused irreversible inactivation of the enzyme *in vivo* in the brain, but not in the pineal (Deguchi and Barchas 1972). Gal, Roggeveen and Millard (1970) suggested that p-CPA might be taken up into the enzyme during its synthesis instead of phenylalanine, and that the new enzyme, with p-CPA incorporated into it, was inactive. Knapp and Mandell (1972 (a)) produced evidence for three mechanisms of p-CPA action.

1) Competition with substrate for entry into the nerve ending.
2) Reversible competitive inhibition of the enzyme for substrate.
3) Irreversible inhibition by incorporation into the enzyme during new protein synthesis in the cell body.

p-CPA produces maximum depletion of brain 5-hydroxytryptamine after two or three days and concentrations of the amine return to normal after thirteen or fourteen days (Koe and Weissman 1966; Knapp and Mandell 1972(a)).

The twenty-four hour or circadian variation of tryptophan-5-hydroxylase has not been widely investigated, but Renson (1973 (b)) was not able to detect a circadian rhythm of tryptophan-5-hydroxylase activity.

The methods most widely used for the estimation of tryptophan-5-hydroxylase activity in the brain utilise a radioactive isotope of tryptophan, a brain or brain region homogenate (particulate or supernatant) and one or more co-factors depending on the purity of the enzyme extract. Since it is difficult to measure 5-hydroxy-tryptophan in these samples, a purified 5-hydroxytrophan decarboxylase extract is included, and 5-hydroxytryptamine is measured as the end product. This assumes that the activity of the decarboxylase and its co-factors is consistent, but as will be seen later (Chapter Seven) this is not the case. In the methods used here I have therefore measured the activity of tryptophan-5-hydroxylase in the presence of a decarboxylase inhibitor (NSD.1024), and 5-hydroxytryptophan, as the end product, has been measured. Although the sensitivity of the method is decreased by this approach, it is hoped that a truer reflection of tryptophan-5-hydroxylase activity has been obtained.

5.2. METHODS

The activity of tryptophan-5-hydroxylase was estimated according to the method of Grahame-Smith (1964) with the exclusion of 5-hydroxytryptophan decarboxylase and the estimation of 5-hydroxytryptamine concentrations. This assay is dependent on the inhibition of 5-hydroxytryptophan decarboxylase using NSD.1024 (3-hydroxybenzyloxyamine). 5-hydroxytryptophan concentrations were determined by chromatographic methods.

Male Sprague Dawley rats (120 - 140g) were maintained for ten days as described in Chapter Two. The rats were sacrificed at 01.00 h. and 13.00 h. and the brains removed, weighed and homogenised in ice-cold Krebs-Ringer solution pH 7.4 (10 ml./g.) as described previously. The homogenate was centrifuged at 500g. at 4°C for ten minutes. 0.8 ml. of the supernatant was pre-incubated for ten minutes in a shaking water bath at 37°C. with 0.1 ml. NSD.1024 2.5×10^{-3}M. 0.1 ml. DL-3-^{14}C tryptophan 57mCi/m.mole 1×10^{-4}M was added, the tubes flushed with O_2 : CO_2 (95:5) and stoppered, and incubated in a shaking water bath for one hour at 37°C. Control homogenates were first boiled for five minutes at 100°C and were then carried through the rest of the procedure.

At the end of incubation 0.1 ml. non-radioactive DL-5-hydroxytryptophan (Sigma) 2×10^{-2}M was added to each tube, and the samples deproteinised by boiling for 5 mins. at 100°C. The samples were centrifuged at 3,000g. to remove the protein precipitate. The supernatant was adjusted to pH 4.0 with 0.1N HCl and 0.25g. of charcoal deactivated with 4% stearic acid (Asatoor and Dalgleish 1956) was added. The samples were shaken for thirty minutes and left to stand for ten minutes. The charcoal was washed with 10 ml. water and the indoles eluted with 10 mls. 7.2% phenol solution, and the eluate evaporated to

dryness. The residue was dissolved in 0.1 ml. water and subjected to two dimensional ascending paper chromatography on Whatmann 3 mm. chromatography paper, the first solvent being propan-2-ol: ammonia (sp.gr. 0.880): water (200:10:20 v/v), the second solvent was butan-1-ol: glacial acetic acid: water (120:30:50 v/v). The sample chromatograms were compared to a standard chromatogram of tryptophan and 5-hydroxytryptophan stained with Ehrlich's reagent (10 ml. of a solution containing 10% ($^{W}/_{v}$) p-dimethylaminobenzaldehyde in conc. HCl plus 40 ml. acetone). The area containing 5-hydroxytryptophan was cut from the chromatogram and slowly eluted with 10 ml. water. The eluate was evaporated to dryness, redissolved in 0.5 ml. water, and 0.4 ml. of this solution was added to 5 ml Unisolve and taken for liquid scintillation analysis.

In other experiments attempts were made to find a soluble enzyme fraction which gave more reproducible results. The initial homogenate was centrifuged at 12,500 g. for thirty minutes and 0.8 ml. of the supernatant was taken for incubation as described previously. After incubation, the incubation mixture was deproteinised by heating to 100°C for five minutes. The mixture was centrifuged for ten minutes at 5,000g, and the supernatant subjected to two dimensional chromatography as described previously.

The effects of $DMPH_4$ (2.7×10^{-4}M) on the activity of tryptophan-5-hydroxylase were assessed using this latter system.

The effects of the inhibition of tryptophan-5-hydroxylase, by p-CPA, on the twenty-four hour rhythm of 5-hydroxytryptamine was assessed as follows:

Male Sprague-Dawley rats (120-140g) were maintained as described previously. Following ten days of acclimatisation, groups of rats were taken at 01.00 h. and 13.00 h. and injected orally either with

p-CPA (316 mg/kg.) or with 0.9% saline. These latter groups served as controls. Rats in groups of seven or eight were sacrificed after 1,2,3,4,7 and 14 days at each clock hour, and the concentration of 5-hydroxytryptamine in the brains was determined for control and experimental groups as described previously.

5.3. RESULTS AND DISCUSSION

The accumulation of ^{14}C-5-hydroxytryptophan after incubation for one hour at 37^{o}C was measured, and calculated as c.p.m. per gram of brain wet weight. The results can be seen in Table 2. The results for the soluble enzyme extract and the effects of $DMPH_4$ on the system are shown in Table 3. The inhibition of tryptophan-5-hydroxylase by p-CPA at 01.00 h. is shown in Figure 32, and at 13.00 h. in Figure 33, and the maximum effects of p-CPA after 3 days is compared with the twenty-four hour rhythm of 5-hydroxytryptamine in the brain in Figure 34.

A typical chromatogram from the assay of 5-hydroxytryptophan is shown in Figure 35. Other indoleamine standards and a mixture of these standards are included to demonstrate the separation of 5-hydroxytryptophan from other indoleamines which may be involved in the assay.

Where $Rf_{(1)}$ are the values obtained from the propan-2-ol: ammonia:water solvent, and $Rf_{(2)}$ are the values obtained from the butan-1-ol:glacial acetic acid: water solvent.

Clock hour	^{14}C-5-HTP (c.p.m./g.)	^{14}C-Tryptophan inoculum (c.p.m.)	% conversion	$DMPH_4$. ^{14}C-5-HTP (c.p.m.)	% conversion
01.oo	1133.9 ± 38.2 (15)	111270	1.02	1186.2	1.07
13.oo	1284.7 ± 15.3 (15)	110800	1.16	1291.7	1.17

Table 3. The conversion of DL-^{14}C- tryptophan to ^{14}C- 5-hydroxytryptophan, and the effects of $DMPH_4$ on enzyme activity in a partially purified homogenate system. The number of determinations is shown in parentheses.

Clock hour.	^{14}C-5-HTP (c.p.m./g.)	^{14}C-Tryptophan inoculum (c.p.m.)	% conversion
01.oo	873.2 ± 28.4 (15)	111002	0.79
13.oo	926.9 ± 36.4 (15)	109989	0.84

Table 2. The conversion of DL-^{14}C-tryptophan to ^{14}C-5-hydroxytryptophan in a crude enzyme preparation. The number of determinations is shown in parentheses.

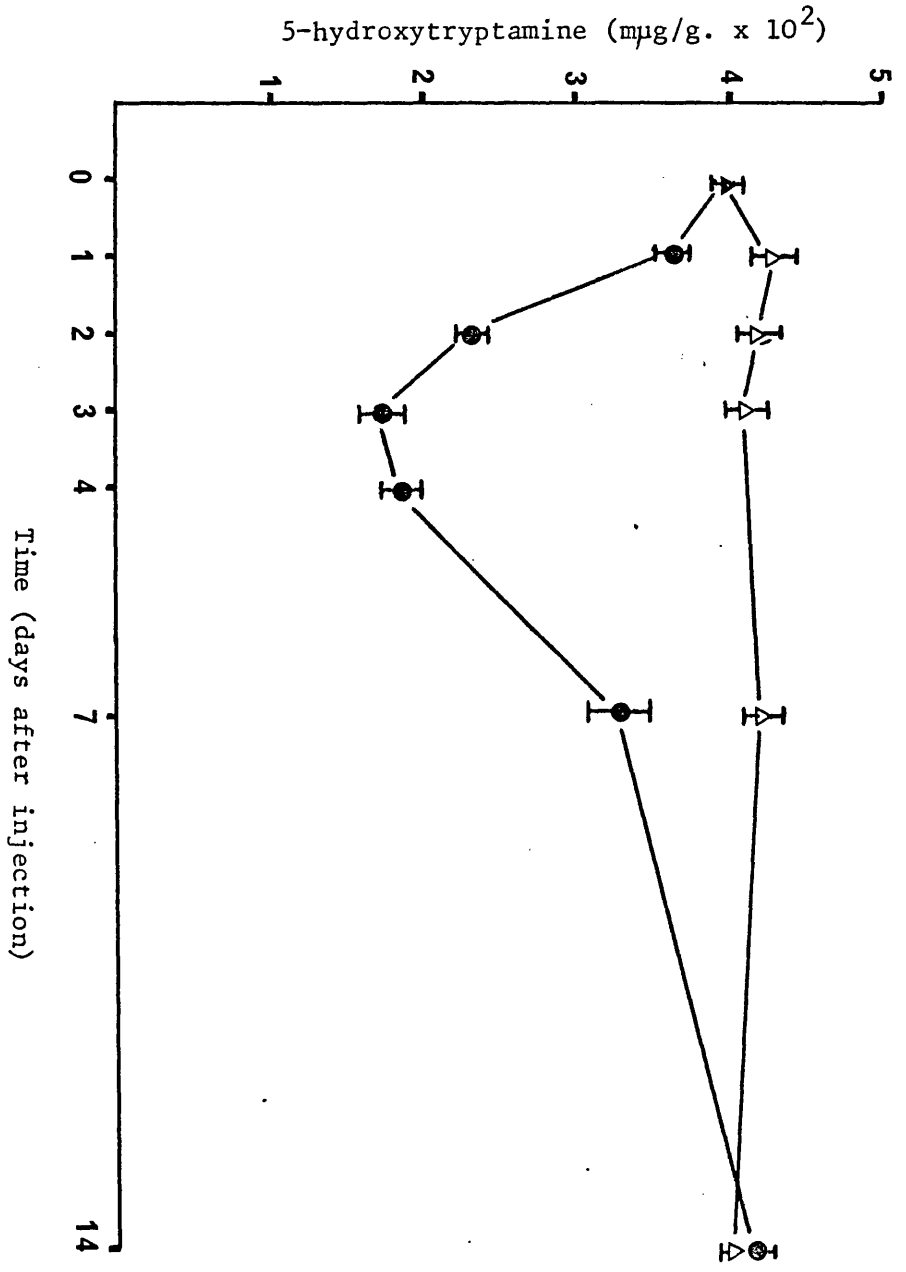


Figure 32. The effects of one injection of p-CPA (316 mg/kg.p.o.) (● —— ●) on 5-hydroxytryptamine concentrations in the rat brain, at 13.00 h. plotted against time (days) Saline control (△ —— △).

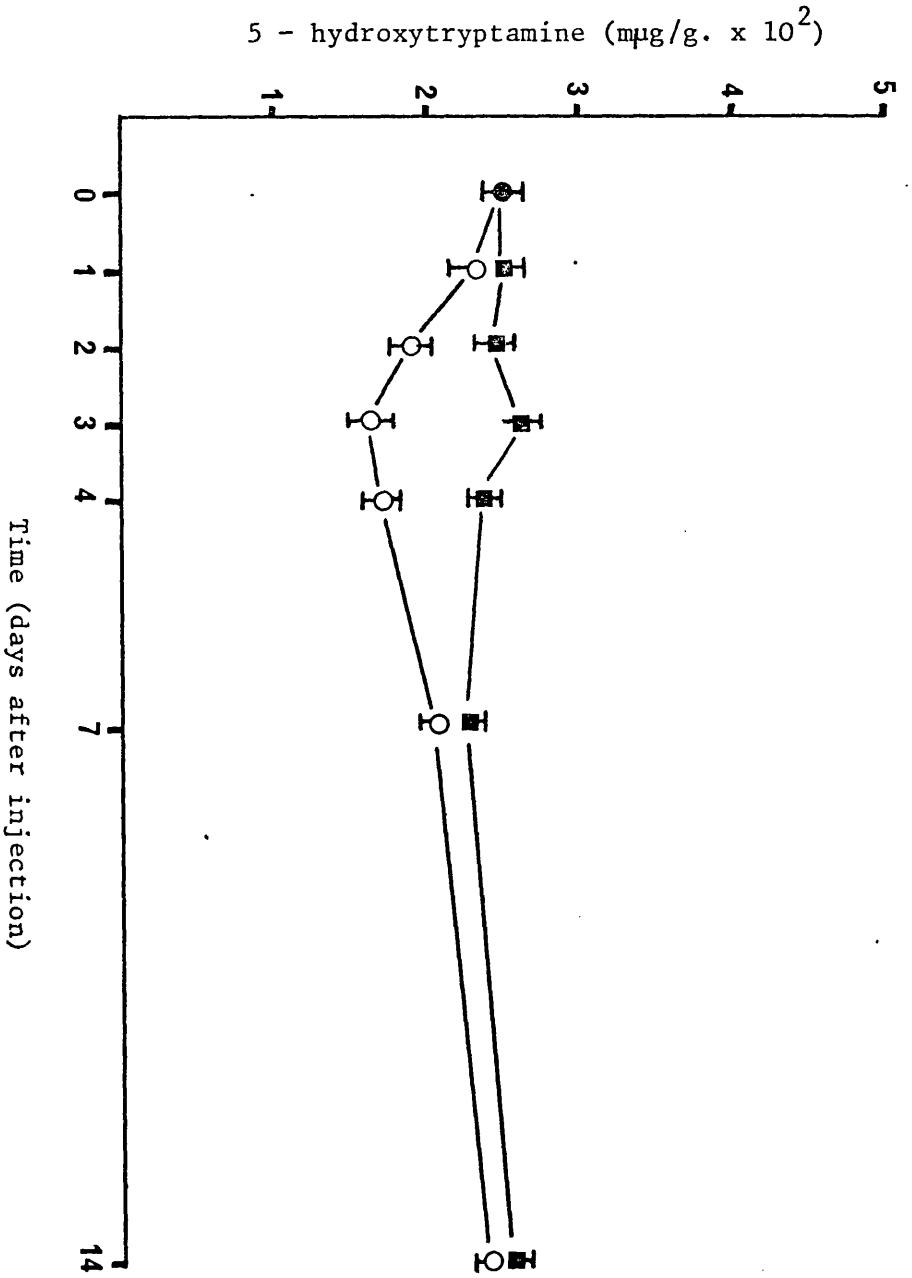


Figure 33. The effects of one injection of p-CPA (316 mg/kg.p.o.) (O—— O) on 5-hydroxytryptamine concentrations in the rat brain at 01.00 h. plotted against time (days). Saline controls (■—— ■).

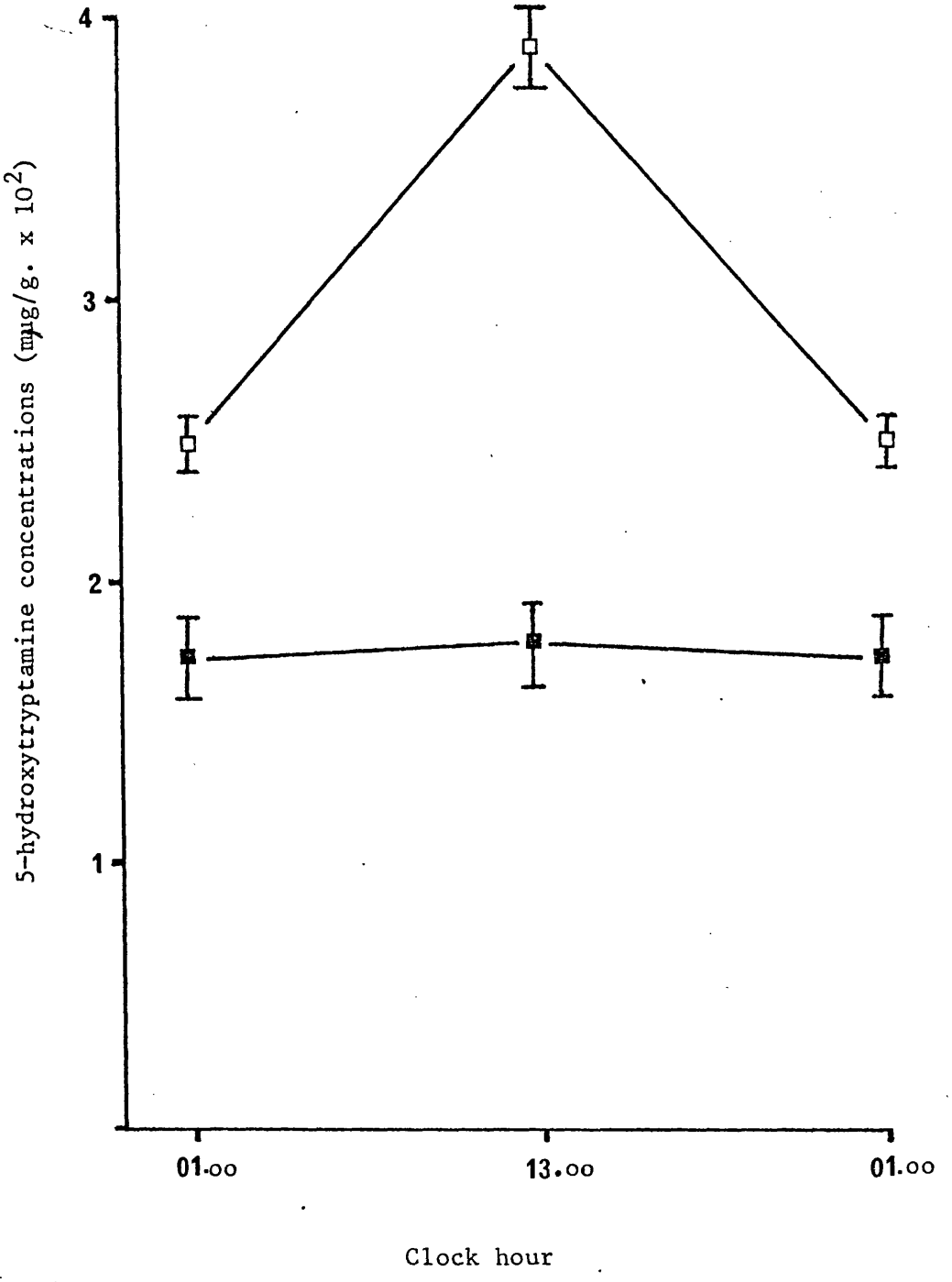


Figure 34. The effects of p-CPA (316 mg/kg.p.o.) on the twenty-four hour variation of 5-hydroxytryptamine concentrations in the rat brain. Rats were killed three days after injection.

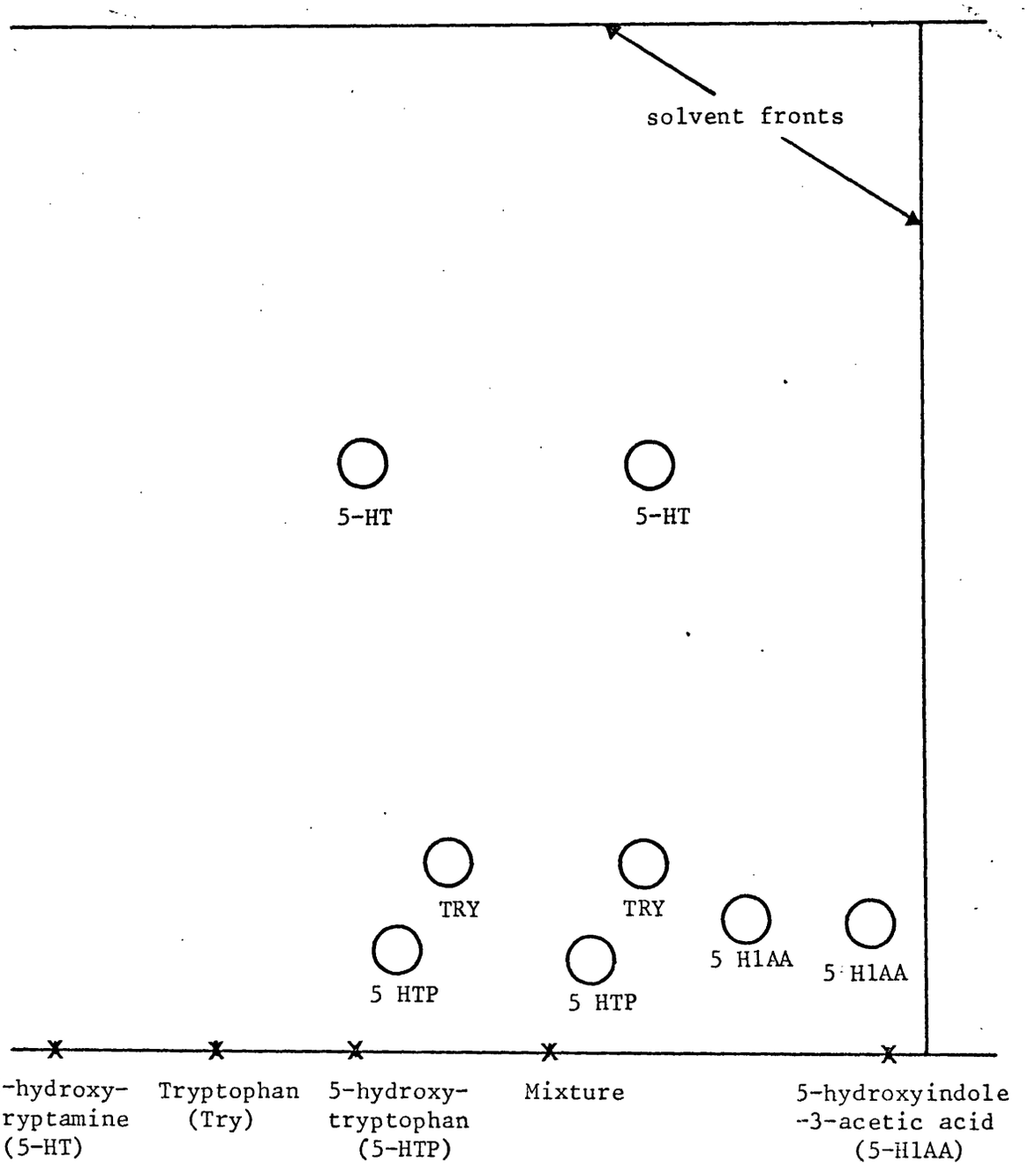


Figure 35. Two dimensional chromatographic separation of Indoles isolated from the rat brain. 1st phase ↑ propan-2-ol: ammonia: water (200:10:20) 2nd phase → butan-1-ol: acetic acid:water (120:30:50).

The Rf values for the first and second solvents were:

Indoleamine	$Rf_{(1)}$	$Rf_{(2)}$
Tryptophan	0.805	0.620
5-hydroxytryptophan	0.902	0.800
5-hydroxytryptamine	0.377	0.590
5-hydroxyindole-3-acetic acid	0.842	0.320

It is quite clear that 5-hydroxytryptophan is well separated from other indoleamines by this method and that the tryptophan substrate did not interfere with the 5-hydroxytryptophan samples taken.

The conversion of DL-^{14}C-tryptophan to ^{14}C-5-hydroxytryptophan was similar in the two methods used. There was no significant difference between the conversion rates at the two clock hours measured, and $DMPH_4$ was ineffective in increasing the activity of tryptophan-5-hydroxylase in these systems. It must be added that I had difficulty in assaying this enzyme, no matter which of the currently popular methods I attempted. Indeed the methods presented here, although apparently crude, were the only methods which showed any degree of reproducibility, but as can be seen from the standard errors of the mean presented in Tables 2 and 3, even these were not small.

From these measurements there appears no doubt that tryptophan-5-hydroxylase is present in the brain of the rat, although the conversion of DL-^{14}C-tryptophan to ^{14}C-5-hydroxytryptophan was slow (0.79 - 1.16% per hour), and that the enzyme does not display a twenty-four hour

rhythm. Since the addition of $DMPH_4$ did not increase the activity of the enzyme, it is assumed that there was already sufficient co-factor present in the homogenate, and that the enzyme was saturated with co-factor.

It has already been shown that a significant twenty-four hour variation of the uptake of tryptophan into a homogenate does not exist. The specific activity of the ^{14}C-tryptophan available for conversion to ^{14}C-5-hydroxytryptophan by tryptophan-5-hydroxylase will therefore be the same at the two clock hours measured. A slightly faster rate of ^{14}C-5-hydroxytryptophan formation was indicated at 13.00 h. in both forms of homogenate, but there was no statistically significant difference between the activities of tryptophan-5-hydroxylase at the two clock hours.

p-Chlorophenylalanine inhibited the synthesis of 5-hydroxytryptamine at the two clock hours with a maximum inhibition after three days and returning to control concentrations after fourteen days. The inhibition was apparently ineffective in reducing 5-hydroxytryptamine concentrations below 170 mμg/g. at this dose of p-CPA. This may either be due to insufficient p-CPA being available for the complete inactivation of tryptophan-5-hydroxylase, or one might suggest the presence of a stable pool of 5-hydroxytryptamine in nerve endings, which cannot be depleted by inhibition of synthesis of the amine. Alternatively 5-hydroxytryptophan taken up from the periphery may account for this low concentration.

p-CPA abolished the twenty-four rhythm of 5-hydroxytryptamine concentrations. Since this inhibition of synthesis was much greater (61%) at 13.00 h. than at 01.00 h. (33%), it is proposed that the twenty-four hour rhythm of 5-hydroxytryptamine concentrations in the rat brain is controlled at some stage in the synthesis of the amine.

Tryptophan-5-hydroxylase has often been considered to be the limiting factor in 5-hydroxytryptamine synthesis. These results suggest that the enzyme is not the regulating factor in the control of the twenty-four hour rhythm of 5-hydroxytryptamine.

If one looks collectively at the results produced in this thesis so far, it is apparent that the total concentration of tryptophan in the brain does not regulate the concentration of 5-hydroxytryptamine, since their respective twenty-four hour rhythms are twelve hours (180^{o}) out of phase. The measurement of the uptake of tryptophan into a synaptosomal fraction showed that this process was independent of total tryptophan concentrations in the brain, but may be dependent on the concentration of tryptophan which is immediately available for uptake into the nerve endings or nerve cell body. It is now apparent that the conversion of tryptophan to 5-hydroxytryptophan is also independent of tryptophan concentration. From these data it is possible to speculate that at different clock hours, the concentrations of 5-hydroxytryptophan in the brain are equal, and should show no twenty-four hour variation unless the concentration is affected by the uptake of the 5-hydroxy amino acid from peripheral sources. At this stage one must conclude that these initial steps in the synthesis of 5-hydroxytryptamine play no direct role in the production of the twenty-four hour rhythm of 5-hydroxytryptamine concentrations. However, a contribution to the establishment of the rhythm is apparent i.e. if one now looks at the twenty-four hour rhythm of 5-hydroxytryptamine concentrations in the brain with highest concentrations at 13.00 h., it is no longer necessary to propose a system whereby tryptophan concentrations, which are twelve hours out of phase with 5-hydroxytryptamine concentrations, might participate in the control

of the twenty-four hour rhythm of 5-hydroxytryptamine concentrations, since the concentration of 5-hydroxytryptophan produced by tryptophan-5-hydroxylase activity is the same at both clock hours.

C H A P T E R S I X

The Role of 5-hydroxytryptophan in the Twenty-four hour rhythm of 5-hydroxytryptamine in the rat brain.

6.1 INTRODUCTION

The conversion of tryptophan to 5-hydroxytryptamine requires aromatic ring hydroxylation in the 5 position and decarboxylation of the side chain. This may occur by either the initial decarboxylation to tryptamine followed by hydroxylation, or hydroxylation to 5-hydroxytryptophan followed by decarboxylation. Udenfriend, Creveling, Posner, Redfield, Daly and Witkop (1959) found no evidence for tryptamine as a precursor of 5-hydroxytryptamine, while the injection of 5-hydroxytryptophan increased brain 5-hydroxytryptamine concentrations (Udenfriend, Titus, Weissbach and Peterson 1956).

5-hydroxytryptophan is normally undetectable in the rat brain, and assay is thus non-productive unless the amino acid is allowed to accumulate following the administration of a 5-hydroxytryptophan decarboxylase inhibitor (Carlsson and Lindqvist 1970). These workers then found that 5-hydroxytryptophan and 5-hydroxytryptamine were similarly distributed, and that the precursor was located in nerve cell bodies and nerve terminals in areas normally containing 5-hydroxytryptamine in the rat brain. Fuxe (1965) and Corrodi, Fuxe and Hokfelt (1967) demonstrated that 5-hydroxytryptophan was specifically taken up and decarboxylated in 5-hydroxytryptamine-containing neurones in the rat brain, while catecholamine-containing neurones were little affected by the amino acid.

L-5-hydroxytryptophan is the active isomer although the D-form may be oxidised and transaminated to the L-isomer (Oates and Sjoerdsma 1961) and may then be taken up into the brain. This uptake was much more rapid in rats between one and twenty-one days of age than in older animals (Kellogg and Lundborg 1972).

The *in vitro* uptake of 5-hydroxytryptophan into nerve endings or cell bodies is an active process requiring glucose and is inhibited by dinitro phenol (Schanberg 1962, Smith 1963). Only a small proportion of the 5-hydroxytryptophan taken up was decarboxylated to 5-hydroxytryptamine. The uptake process was inhibited by L-phenylalanine and α-methyl dopa, and was not linked to decarboxylation (Smith 1963).

The behavioural responses of rats which have been injected with high doses of 5-hydroxytryptophan are very marked, characteristically producing an overt stimulation (piloerection, panting increased motor activity) (Brodie, Comer, Costa and Dlabac 1966). These authors suggested that this was due to the release of noradrenaline by 5-hydroxytryptophan since one large dose of the amino acid reduced brain noradrenaline concentrations by 50%. Johnson, Kim and Boukma (1968) demonstrated the inhibition of the synthesis of noradrenaline by 5-hydroxytryptophan (600 mg/kg i.p.) and in addition showed that the incorporation of ^{14}C-tyrosine into rat brain noradrenaline and the specific activity of the isolated noradrenaline were reduced. They found no evidence that 5-hydroxytryptophan mediated the release of noradrenaline.

5-hydroxytryptophan has been used in clinical trials to alleviate the symptoms of schizophrenia and depression, although the results are rarely encouraging (Wyatt, Vaughan, Kaplan, Galanter and Green 1973; Brodie, Sack and Siever 1973). 5-hydroxytryptophan has however been shown to increase slow wave sleep and to delay paradoxical sleep in rabbits (Tabushi, Kaoru and Himwich 1970), so might therefore be expected to aid schizophrenic and depressed patients.

Weiss, Wurtman and Munro (1973) showed that 5-hydroxytryptophan was able to disaggregate brain polysomes, and they therefore postulated that intracellular amines may normally participate in the control of brain protein synthesis.

In this Chapter the concentrations of 5-hydroxytryptophan have been estimated over a twenty-four hour period in the rat brain, and characteristics of the uptake of DL-^{14}C-5-hydroxytryptophan into homogenates of the septal region of rat brain have been determined. The effects of the results obtained on the twenty-four hour rhythm of 5-hydroxytryptamine are discussed.

6.2 METHODS

Male Sprague Dawley rats (120 - 140g) were maintained for ten days under constant environmental conditions (Chapter Two).

6.2.1 Determination of the Twenty-four hour rhythm of 5-hydroxytryptophan concentrations in the rat brain.

Following acclimatisation, rats in groups of seven or eight were killed by decapitation at four-hourly intervals commencing at 09.00 h. The pineal glands were discarded and the brains were immediately removed and 5-hydroxytryptophan was isolated by the chromotographic methods described in Chapter Five. No 5-hydroxytryptophan was added to brain homogenates, only to standard chromatograms. The areas containing 5-hydroxytryptophan were cut out and the amino acid was eluted from the paper with 10 ml. distilled water evaporated to dryness under reduced pressure at 55°C and made up to 2.0 ml. with distilled water. 1.0 ml. was acidified with 0.3 ml. conc. HCl and the native fluorescence of 5-hydroxytryptophan was measured at excitation wavelength 280 mμ and emission wavelength 540 mμ (wavelengths uncorrected). The fluorescence spectrum of the amino acid was determined.

6.2.2. Determination of the Uptake of DL-^{14}C-5-hydroxytryptophan into homogenates of the Septal Region of rat brain.

The method was exactly the same as that described in Chapter Three for the uptake of DL-^{14}C-5-hydroxytryptamine into homogenates of the septal region of rat brain with the exception that DL-^{14}C-5-hydroxytryptophan (51 mCi/mmole) was used as the substrate. The Michaelis

constant and the effects of time, temperature and pH on the uptake of DL-^{14}C-5-hydroxytryptophan were determined at 01.00 h. and 13.00 h. by the methods described previously in Chapter Three. All results are the means of three experiments.

6.3 RESULTS and DISCUSSIONS

The fluorescence spectrum of 5-hydroxytryptophan exibited two peaks (Figure 36) when measured by the native fluorescence method. The first, at excitation wavelength 280 mμ, emission wavelength 540 mμ, and the second at excitation wavelength 300 mμ and emission wavelength 580 mμ. When fluorescence intensity was plotted against the concentration of 5-hydroxytryptophan (Figure 37) a linear plot was obtained only at the former wavelengths which were therefore selected to measure the concentrations of 5-hydroxytryptophan from the chromatograms.

The effects of the related indoleamines, tryptophan, 5-hydroxytryptamine and 5-hydroxyindole-3-acetic acid, on the native fluorescence of 5-hydroxytryptophan were determined (Table 4).

Indoleamine (1.0 ml (300mμg/ml) + 0.3 ml.HCl)	Fluorescence units.(x10^3)
Tryptophan	0.126
5-hydroxytryptophan	0.350
5-hydroxytryptamine	0.355
5-hydroxyindole-3-acetic acid	0.505
HCl + water blank	0.126

Table 4. The effects of related indoleamines on the native fluorescence of 5-hydroxytryptophan. Each measurement is the mean of four determinations.

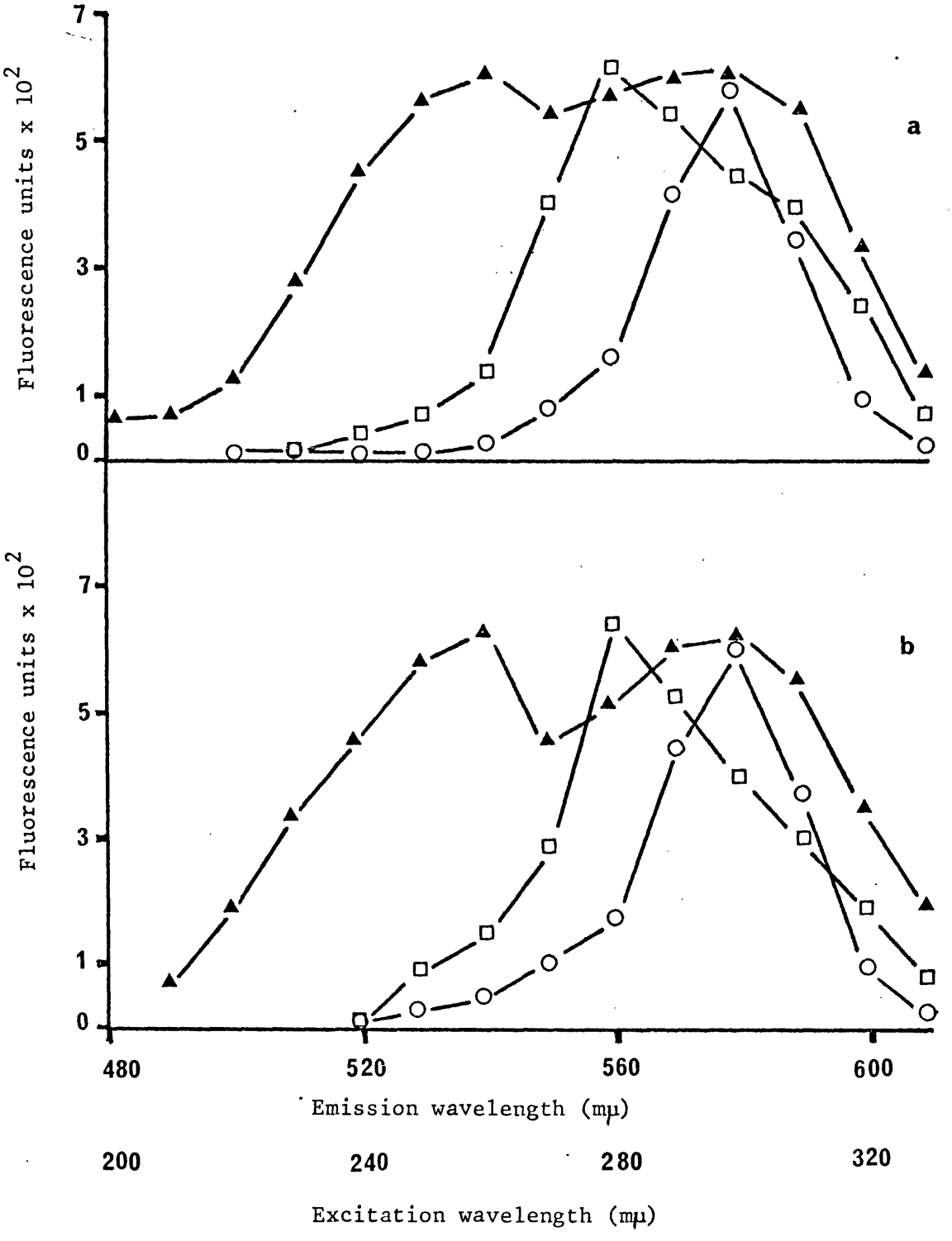


Figure 36. The fluorescence spectrum of extracted 5-hydroxytryptophan (a) compared with the pure compound (b). (▲——▲) excitation 280 mμ scan emission; (□——□) emission 540 mμ scan excitation; (○——○) emission 580 mμ scan excitation.

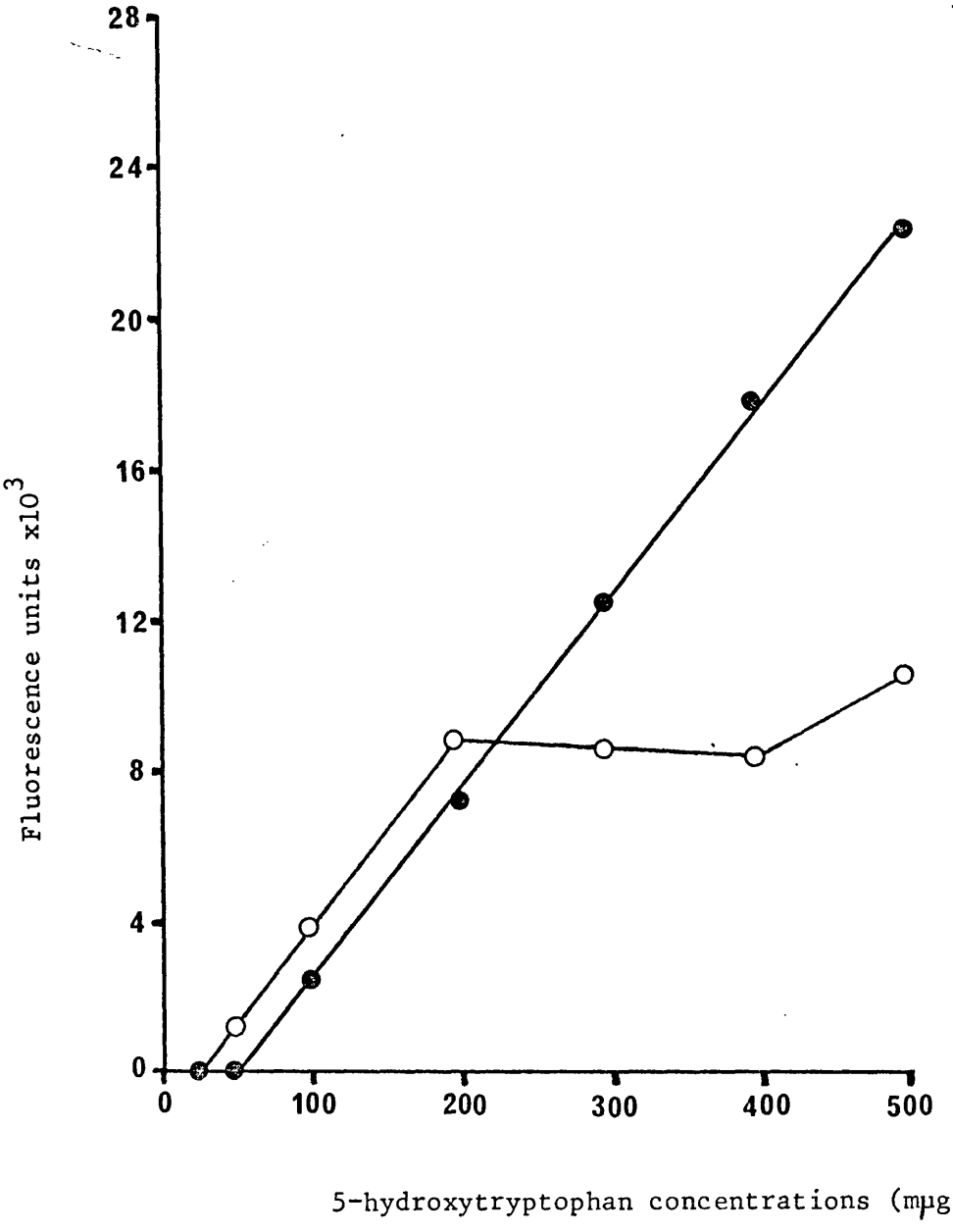


Figure 37. Standard curves of 5-hydroxytryptophan fluorescence at wavelengths 280 mμ, 540 mμ (● —— ●) and 300 mμ, 580 mμ (○ —— ○).

It was found that although tryptophan has no effect, both 5-hydroxytryptamine and 5-hydroxyindole-3-acetic acid fluoresce at least as much as 5-hydroxytryptophan at these wavelengths. It was therefore found to be essential to separate the indoleamines before measurement, and this has already been shown to be accomplished by the chromatographic method employed (Chapter Five).

The concentrations of 5-hydroxytryptophan determined over a twenty-four hour period can be seen in Table 5. The results shown are those calculated from the total 5-hydroxytryptophan measured with the water blank substracted, and are expressed as mμg.5-hydroxytryptophan per g. brain wet weight.

Clock hour	5-hydroxytryptophan (mμg./g.brain)
09.00	65 ± 4.2 (7)
13.00	72 ± 3.9 (8)
17.00	55 ± 5.1 (7)
21.00	77 ± 6.1 (7)
01.00	62 ± 3.6 (8)
05.00	52 ± 3.6 (7)
09.00	59 ± 4.1 (8)

Table 5. The twenty-four hour variation of 5-hydroxytryptophan concentrations in the rat brain. Numbers of animals used in each group are shown in brackets.

These results can only be regarded as estimates of 5-hydroxytryptophan concentrations since the fluorimetric measurements obtained were only marginally greater than readings secured for the water blank. From the results obtained, the twenty-four hour variation of 5-hydroxytryptophan concentrations in the rat brain did not correspond to a sine curve when calculated by Fourier analysis, and it must therefore be concluded that there was no twenty-four hour rhythm of 5-hydroxytryptophan concentrations in the rat brain. However, a method providing better resolution of the extracted product might indicate differences not detected here. Alternatively, the use of decarboxylase inhibitors may give an indication of endogenous concentrations of the amino acid, although it was felt that a method which measured endogenous concentrations directly was essential to this project. In any event the very low concentrations of 5-hydroxytryptophan recorded led to the inevitable conclusion that there is no store of the amino acid in the rat brain, but that it must be rapidly broken down to 5-hydroxytryptamine or via other routes.

The uptake of DL-^{14}C-5-hydroxytryptophan into septal homogenates increased with time to a maximum after six minutes, remained stable up to twenty minutes and slowly declined after this period when incubated at 37^{o}C at pH 8.0 (Figure 38).

The uptake process was temperature (Figure 39) and pH (Figure 40) dependent and there was no significant difference between the uptake process subjected to these variables at 01.00 h. and 13.00 h. Maximum uptake was obtained at 37^{o}C and pH 8.0 at both clock hours.

Since the endogenous concentrations of 5-hydroxytryptophan are apparently the same at 01.00 h. and 13.00 h., it can be assumed that the specific activity of the added DL-^{14}C-5-hydroxytyptophan is the

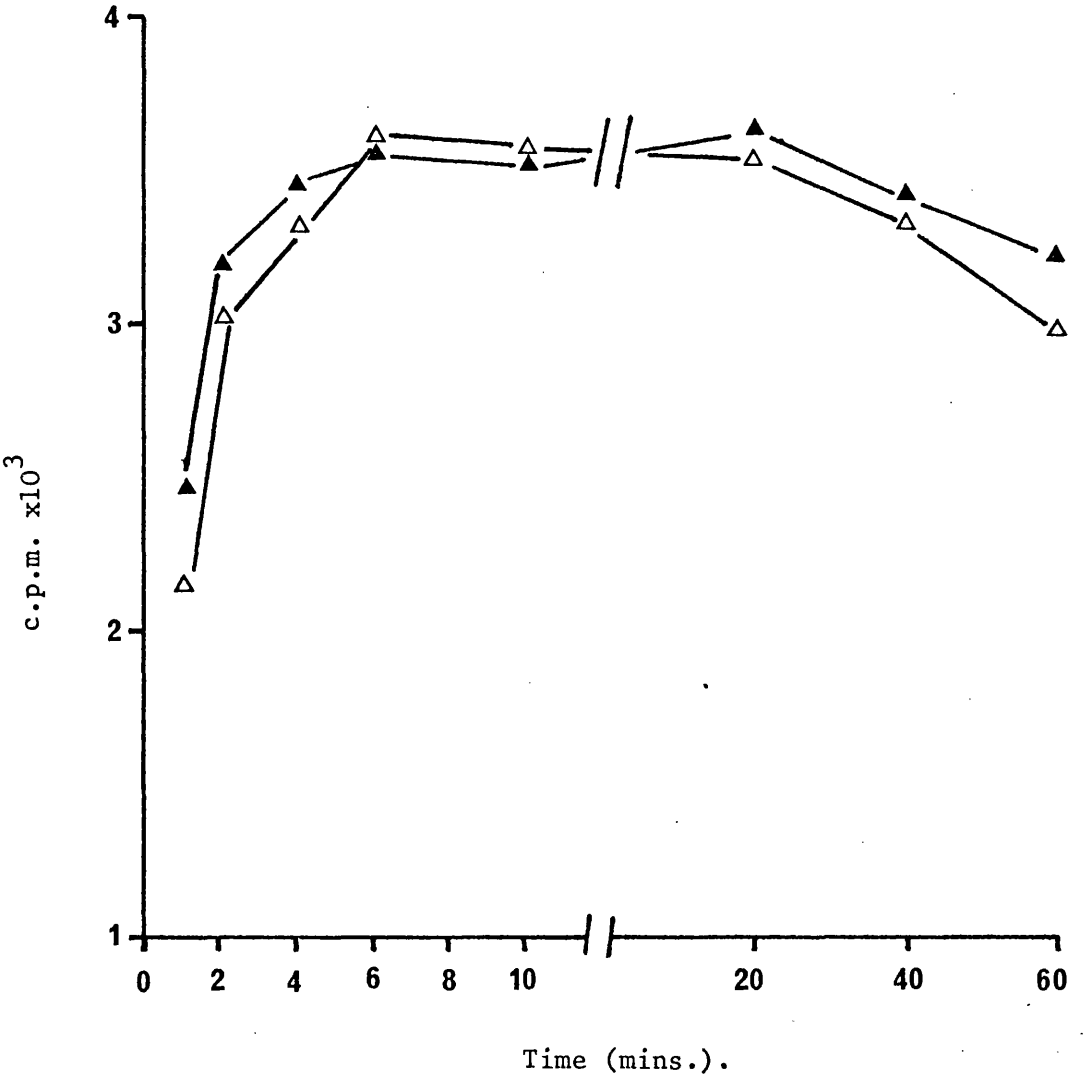


Figure 38. The uptake of ^{14}C-5-hydroxytryptophan (5×10^{-6} M) into a septal homogenate plotted against incubation time at 37°C, pH 8.0. 13.00 h. (△——△) and 01.00 h. (▲——▲).

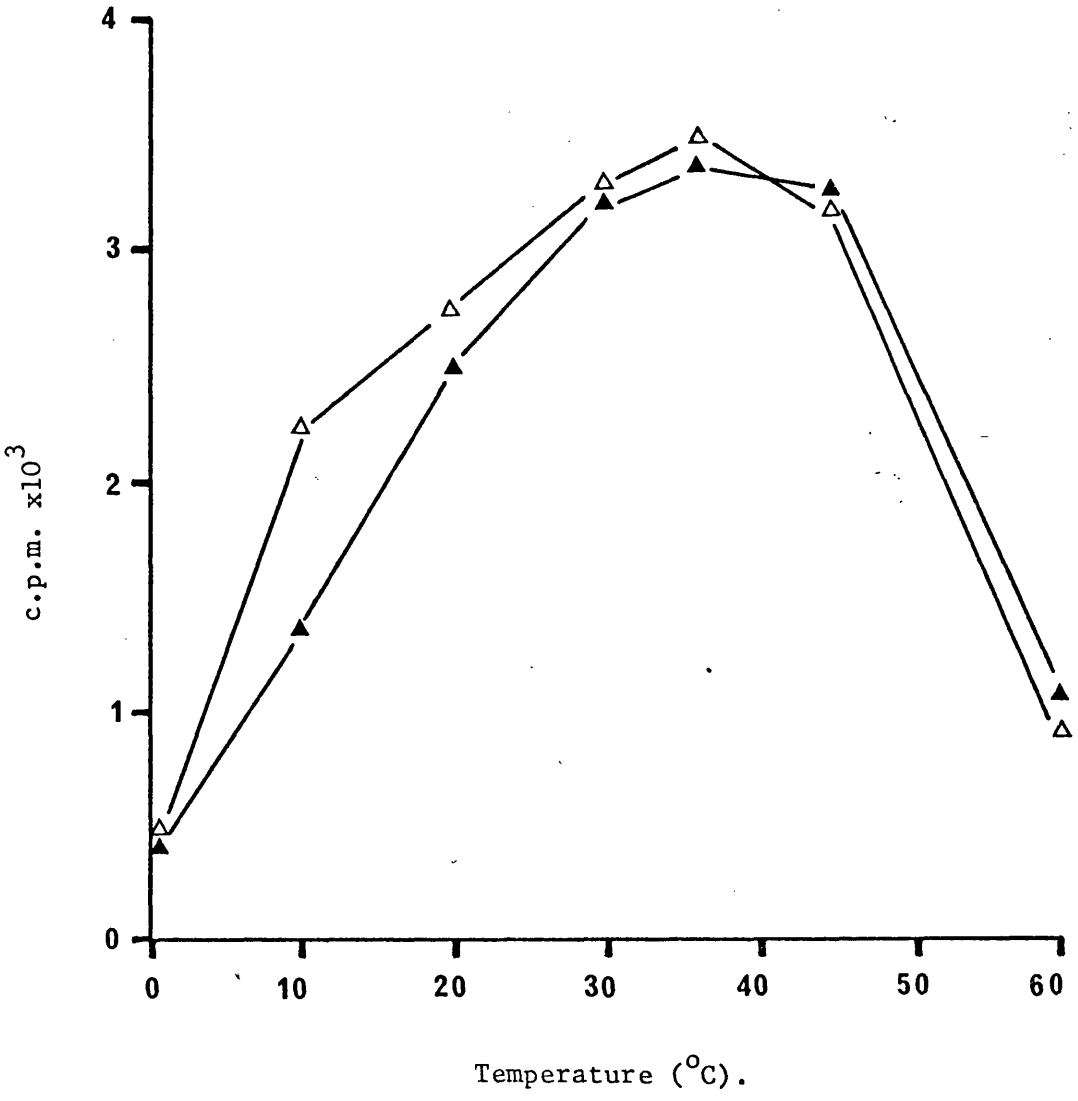


Figure 39. The uptake of ^{14}C-5-hydroxytryptophan (5×10^{-6}M) into a septal homogenate plotted against temperature at pH 8.0. 13.00 h (△——△) and 01.00 h. (▲——▲). Incubation time was 6 minutes.

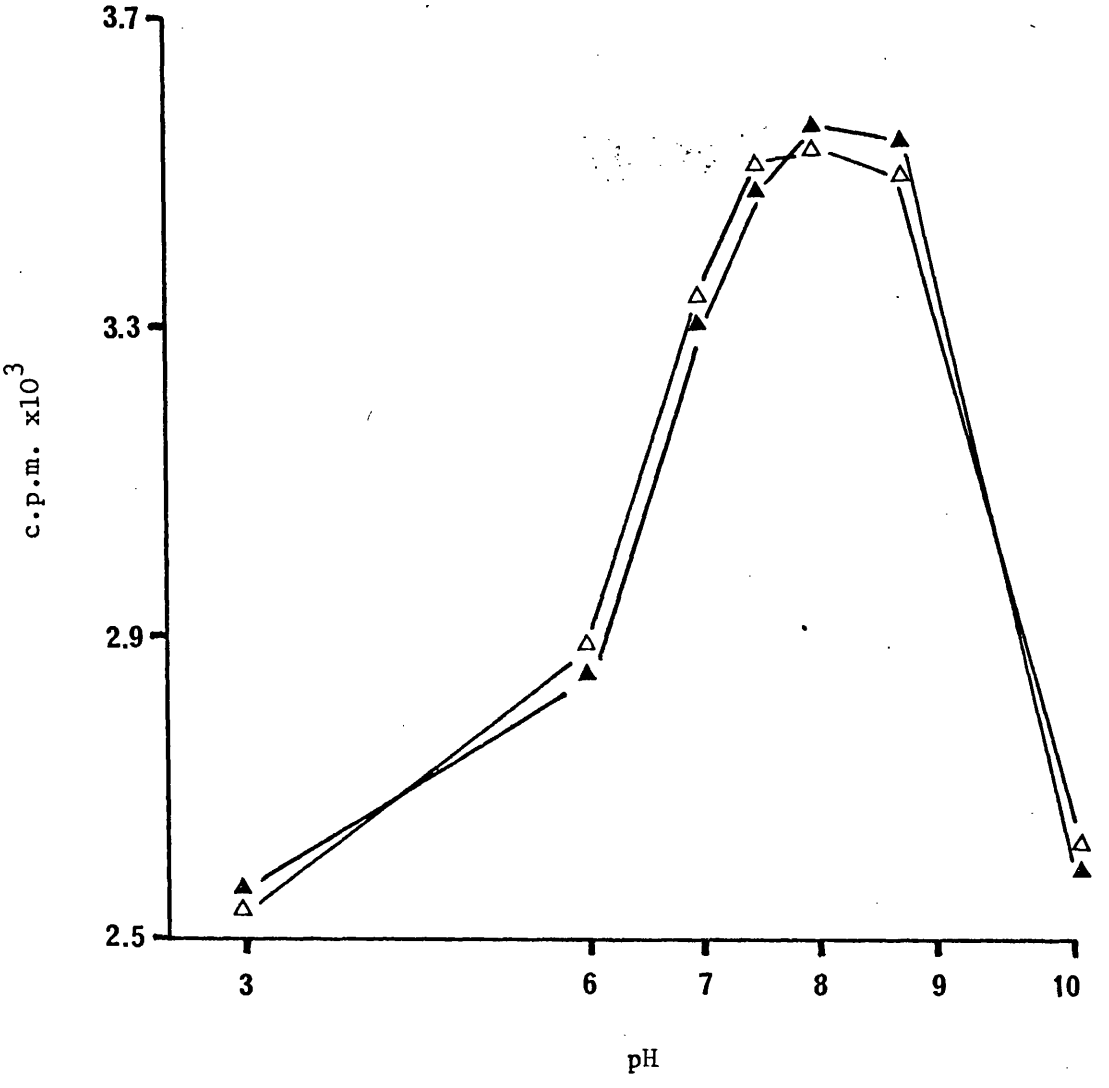


Figure 40. The uptake of ^{14}C-5-hydroxytryptophan (5×10^{-6}M) into a septal homogenate plotted against pH at 37°C. Incubation time was 6 minutes. 13.00 h. (Δ——Δ) and 01.00 h. (▲——▲).

same at these clock hours. The Michaelis constant for 5-hydroxy-tryptophan uptake was 8.3×10^{-6}M at 01.00 h. (Figure 41) and 9.3×10^{-6}M at 13.00 h. (Figure 42). There was no significant difference between these values, and there was no significant difference in V_{max} at these clock hours. It was therefore concluded that there was no twenty-four hour rhythm of the uptake of 5-hydroxytryptophan in the rat brain.

It was concluded from these results that neither the endogenous concentrations of 5-hydroxytryptophan nor the uptake of 5-hydroxytryptophan into 5-hydroxytryptamine-containing neurones in the brain play a controlling part in the production of the twenty-four hour rhythm of 5-hydroxytryptamine concentrations in the rat brain.

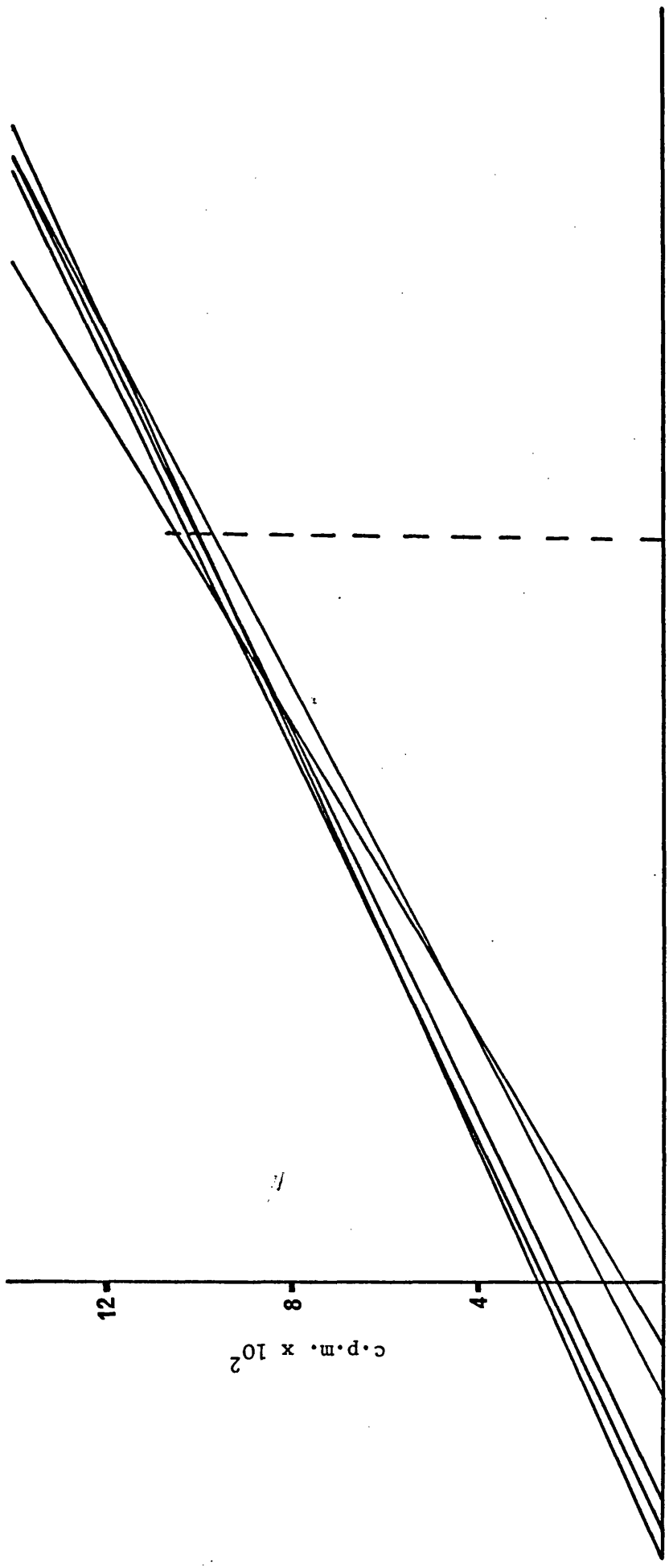


Figure 41. Direct linear plot to determine the Michaelis constant of the uptake of ^{14}C-5-hydroxytryptophan into a septal homogenate at 01.00 h. at 37^{o}C and pH 8.0.

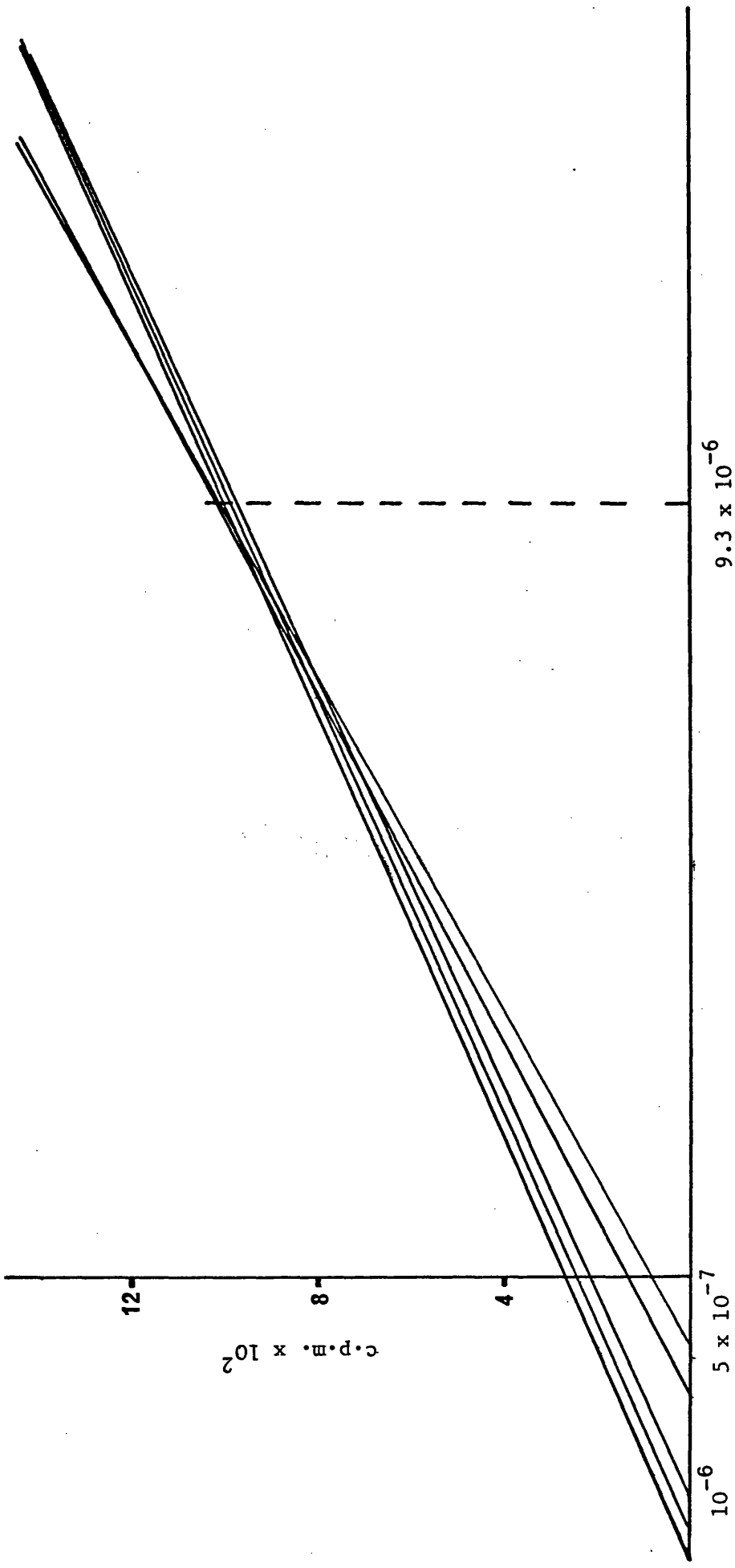


Figure 42. Direct linear plot to determine the Michaelis constant of the uptake of ^{14}C-5-hydroxytryptophan into a septal homogenate at 13.00 h. at 37^{o}C and pH 8,0.

CHAPTER SEVEN

The Involvement of 5-hydroxytryptophan decarboxylase in the production of the Twenty-four Hour Rhythm of 5-hydroxytryptamine in the rat brain.

7.1 INTRODUCTION

5-hydroxytryptophan decarboxylase (EC.4.1.1.28) (M.wt.112,000) catalyses the synthesis of 5-hydroxytryptamine from 5-hydroxytryptophan in the CNS, and the localisation of the enzyme parallels that of 5-hydroxytryptamine (Gaddum and Giarman 1956). There is good evidence that most of the 5-hydroxytryptophan decarboxylase in the rat brain is present in monoamine neurones and is highly active there (Anden, Magnusson and Rosengren 1965).

The trivial name L-aromatic amino acid decarboxylase has been adopted to describe both 5-hydroxytryptophan decarboxylase and 3,4-dihydroxy-L-phenylalanine decarboxylase (EC.4.1.1.26) activities, and there remains some dispute as to whether these enzymes are different. Clark Weissbach and Udenfriend (1954) found two distinct enzymes, although Lovenberg, Weissbach and Udenfriend (1962); Christenson, Dairman and Udenfriend (1970) and Hokfelt, Fuxe and Goldstein (1973) could only find one enzyme, but this had the ability to decarboxylate both 5-hydroxytryptophan and 3,4-dihydroxyphenylalanine. (DOPA). Coulson, Bender and Jepson (1969) found four peaks of decarboxylase activity following separation of the protein fraction one of these was more specific towards the 5-hydroxytryptophan substrate while two others were more specific for 3,4-dihydroxyphenylalanine. Yuwiler, Geller and Eiduson (1960) proposed one enzymic site involved in the decarboxylation of 5-hydroxytryptophan and DOPA and they suggested that DOPA may combine irreversibly at a 5-hydroxytryptophan site while reacting at a different site but 5-hydroxytryptophan probably could not bind irreversibly at DOPA sites.

5-hydroxytryptophan and DOPA show widely different pH optima for the enzyme but they are mutually competitive, and there is apparently no relation between the probable ionisation states of

the substrates and the pH dependence of the enzyme (Bender and Coulson 1972). These authors proposed that the enzyme has a complex active site with separate affinity sites for the two substrates adjacent to a single catalytic site. The decarboxylase enzyme(s) has a pyridoxal phosphate co-factor requirement, and evidence of only one catalytic site has been presented by the finding of only one molecule of pyridoxal phosphate per molecule of enzyme (Christenson, Dairman and Udenfriend 1970).

Aromatic-L-amino acid decarboxylase is a soluble enzyme in most tissues, but in brain preparations a part of the activity is apparently bound or enclosed in vesicles (Arnaiz and DeRobertis 1964). Sims Davis and Bloom (1973) found 5-hydroxytryptophan decarboxylase activity almost equally distributed between soluble and particulate fractions, and there was a definite association of the enzyme with fractions containing large numbers of nerve endings.

Maturation studies of 5-hydroxytryptophan decarboxylase have shown that in all brain regions the enzyme and 5-hydroxytryptamine concentrations mature differently (Baker, Hoff and Smith 1973) thus indicating that the enzyme is not a regulating factor in the synthesis of 5-hydroxytryptamine during maturation. Conversely Hakanson, Lombard, Des Gouttes and Owman (1967) found 5-hydroxytryptophan decarboxylase to be a controlling step in the maturation of 5-hydroxytryptamine concentrations in the pineal gland.

Yuwiler, Geller and Eiduson (1971) were unable to demonstrate a circadian variation for 5-hydroxytryptophan decarboxylase in liver extracts.

In this Chapter 5-hydroxytryptophan decarboxylase activity has been assessed and characteristics of the enzyme have been determined in rat brain homogenates. The activity of the enzyme was also determined in a purified enzyme preparation, and the twenty-four hour variation of enzymic activity was measured in both enzyme preparations.

7.2 METHODS

Male Sprague Dawley rats (120 - 140g) were maintained for ten days under constant environmental conditions before each experiment as described in Chapter Two.

7.2.1 Assay of 5-hydroxytryptophan decarboxylase activity

Rats were killed by decapitation, the pineal glands discarded, and the brains removed immediately weighed and homogenised in ice-cold 0.25M sucrose (4 ml./brain). The homogenates were centrifuged at 1,500g. for ten minutes at 4°C. 0.1 ml. pargyline (10^{-3}M made up in pH 8.0 phosphate buffer 94.5 ml.) Na_2HPO_4 (1.780g/100 ml) + 5.5 ml KH_2PO_4 (1.361g/100 ml)), and 0.1 ml. pyridoxal phosphate (4×10^{-4}M made up in pH 8.0 phosphate buffer) were added to 1.0 ml. of the supernatant. The mixture was pre-incubated at 37°C for ten minutes. 1.0 ml. DL-5-hydroxytryptophan (5×10^{-3}M made up in pH 8.0 phosphate buffer) was added and these reactants were incubated together for varying time intervals. The reaction was stopped by the addition of 0.1 ml. 4N.H_2SO_4. The 5-hydroxytryptamine formed from the reaction was assayed by the method of Snyder, Axelrod and Zweig(1965) as described in Chapter Three with the exception that the borate buffer pH 10.0 washing was repeated three times.

In later experiments DL-^{14}C-5-hydroxytryptophan (2.5×10^{-5}M) was used as the substrate. The incubation was carried out as already described but the assay of 5-hydroxytryptamine was modified as follows:

The incubation mixture was shaken for five minutes with 10 ml. acid

n-butan-1-ol. 15 ml. water and 30 ml. n-heptane were added and the mixture was shaken for five minutes and centrifuged at 500g. for five minutes. 10 ml. of the aqueous phase was added to 5 ml. borate buffer pH 10.0 10 ml. butan-1-ol and 4 gm. sodium chloride, shaken for five minutes and centrifuged at 500g. for five minutes. All the butan-1-ol phase was shaken twice more with 2.0 ml. borate buffer pH 10.0 and 2g. NaCl. 5 ml. of the butan-1-ol phase was added to 2.0 ml. phosphate buffer pH 7.0 and 10 ml. n-heptane and was shaken for five minutes and centrifuged at 500g. for five minutes. 0.5 ml. phosphate buffer phase was added to 5 ml. Unisolve and taken for liquid scintillation analysis.

The effects of pH, temperature, incubation time and pyridoxal phosphate concentration were determined following one or other of these assay procedures. The conditions for the different experiments presented in this Chapter will be described in the legends to the Figures.

7.2.2 The twenty-four hour variations of 5-hydroxytryptophan-decarboxylase activity.

The twenty-four hour variation of 5-hydroxytryptophan decarboxylase activity was determined using the isotopic method described above. Rats were killed at four-hourly intervals commencing at 09.00 h.

7.2.3 Investigation of factors affecting 5-hydroxytryptophan-decarboxylase activity.

In order to determine whether the activity of the enzyme was a consequence of tryptophan-5-hydroxylase activity or endogenous 5-hydroxytryptophan productions, groups of rats were pre-treated with

p-chlorophenylalanine (316 mg/kg p.o) and killed three days later at varying clock hours, and the activity of 5-hydroxytryptophan decarboxylase was determined isotopically as described before.

In order to determine whether the variation of the activity of the enzyme was dependent upon its co-factor requirements, the enzyme was isolated by the method of Clark, Udenfriend and Weissbach (1954) and its activity was measured as follows:

Rats were killed by decapitation at 01.00 h. and 13.00 h. The pineal glands were discarded and the brains were immediately removed and homogenised in ice-cold distilled water (8 ml./brain). The homogenates from four brains were pooled and centrifuged at 1500 rpm for twenty minutes at $0^{o}C$. 14 ml. saturated ammonium sulphate pH 8.1 was added to the supernatant. The mixture was shaken, allowed to stand for twenty minutes at $0^{o}C$, and was then centrifuged at 1500 rpm for twenty minutes at $0^{o}C$. 14 ml. saturated ammonium sulphate pH 8.1 was added to the supernatant, shaken and allowed to stand for twenty minutes at $0^{o}C$. The mixture was centrifuged at 3000 rpm for thirty minutes at $0^{o}C$ and the precipitate was dissolved in 5 ml. water and dialysed against ice-cold distilled water for three hours. The pH was rapidly adjusted to pH 5.8 with 0.02N acetic acid, an equal amount ($^{w}/v$) of alumina was added and the mixture was allowed to stand for thirty minutes on ice stirring occasionally. The alumina was washed once with 5 ml. water and the washings discarded, and then four times with 5 ml. 0.1M phosphate buffer pH 6.3 and the washings were collected on ice. The activity of the enzyme was assayed as follows: all solutions were made up in 0.1M phosphate buffer pH 8.0.

0.1 ml. pyridoxal phosphate (4 x 10^{-4}M) and 1.3 ml. 0.1M phosphate buffer pH 8.0 were added to 2.0 ml. of the enzyme preparation and the mixture was pre-incubated for ten minutes at 37°C. 0.1 ml DL-5-hydroxytryptophan (1 x 10^{-5}M) was added and the reactants were incubated for 15, 30 and 45 mins. at 37°C. In some tubes 0.1 ml. 0.1M phosphate buffer pH 8.0 replaced 0.1ml DL-5-hydroxytryptophan in order to measure the unreacted blank. The reaction was stopped with 0.1 ml. 4N.H_2SO_4. The 5-hydroxytryptamine formed during the reaction was assayed as follows:

The reaction mixture was adjusted to pH 10.0 with N.NaOH, saturated with NaCl and shaken for five minutes with 10 ml. washed butan-1-ol. 8.5 ml. of the butan-1-ol phase was washed three times with 2.0 ml. salt saturated borate buffer pH 10.0. 7.5 ml. of the organic phase was added to 10 ml. n-heptane and 2.0 ml. phosphate buffer pH.7.0 and shaken for five minutes. 1.2 ml. phosphate buffer was heated at 70°C for thirty minutes with 0.1 ml. 0.1M ninhydrin reagent. The mixture was cooled to 4°C for one hour and the fluorescence measured at excitation wavelength 380 mμ, emission wavelength 490 mμ (wavelengths uncorrected).

7.3 RESULTS AND DISCUSSION

The rate of synthesis of 5-hydroxytryptamine from 5-hydroxytryptophan in a crude homogenate (method 7.2.1) varied at the two clock hours measured (Figure 43). The rate of formation of 5-hydroxytryptamine was 0.875 μ moles/h^{-1}/g^{-1} brain (wet weight) at 13.00 h. and 0.70 μ moles/h^{-1}/g^{-1} at 01.00 h. At 13.00 h synthesis was linear over a three hour period but synthesis remained linear only over a two hour period in samples taken at 01.00 h. and then decreased rapidly.

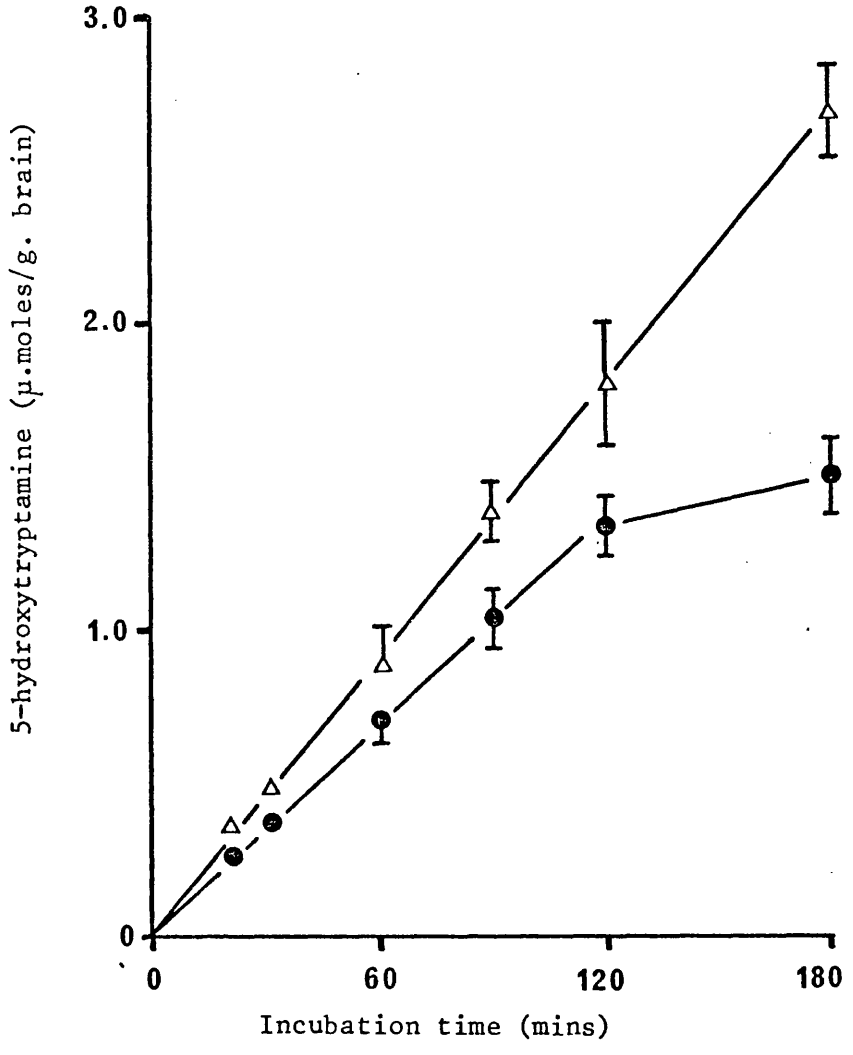


Figure 43. The effects of incubation time on 5-hydroxytryptophan decarboxylase activity at 01.00 h (●——●) and 13.00 h. (△——△) in a homogenate of whole rat brain. 5-hydroxytryptophan 5×10^{-3}M; pH 8.0; 37°C. Results are the mean of five experiments.

At 01.00 h and 13.00 h. the optimum temperature of incubation for 5-hydroxytryptophan decarboxylase was 37°C (Figure 44) and the pH optimum was 8.0 (Figure 45). Activity was very low at high (60°C) and low (4°C) temperatures and in acid (pH 5.0) conditions.

The rate of 5-hydroxytryptamine synthesis was 0.430 μ moles/h^{-1}/g^{-1} at 13.00 h. and 0.320 μ moles/h^{-1}/g^{-1} at 01.00 h. in samples pre-incubated without added pyridoxal phosphate (Figure 46). The rate of synthesis increased with added pyridoxal phosphate concentration in the pre-incubation mixture to a maximum at a concentration of 4×10^{-4}M. The increased rate of synthesis was approximately 100% at both clock hours.

As has already been stated, one molecule of enzyme receives only one molecule of co-factor (Christenson, Dairman and Udenfriend 1970) and it is therefore assumed that the enzyme is, at least in part, maintained in an inactive form since addition of pyridoxal phosphate was able to increase the amount of product formed in a given time. The activity of the enzyme increased proportionally in samples taken at 01.00 h. and 13.00 h. when pyridoxal phosphate was added. As a result it can be concluded that the twenty-four hour variation in enzyme activity is probably not due to different amounts of 5-hydroxytryptophan decarboxylase being present.

In order to determine whether the variation of 5-hydroxytryptophan activity was a consequence of pyridoxal phosphate availability or whether there was a change in the activity of the enzyme at different clock hours, the enzyme was isolated and assayed as described in the Methods in this Chapter (Methods 7.2.3). The rate of 5-hydroxytryptamine synthesis using the purified enzyme extract was maximal after thirty minutes of incubation under the conditions employed, and the rate did not vary between samples taken at 01.00 h. and 13.00 h (Figure 47).

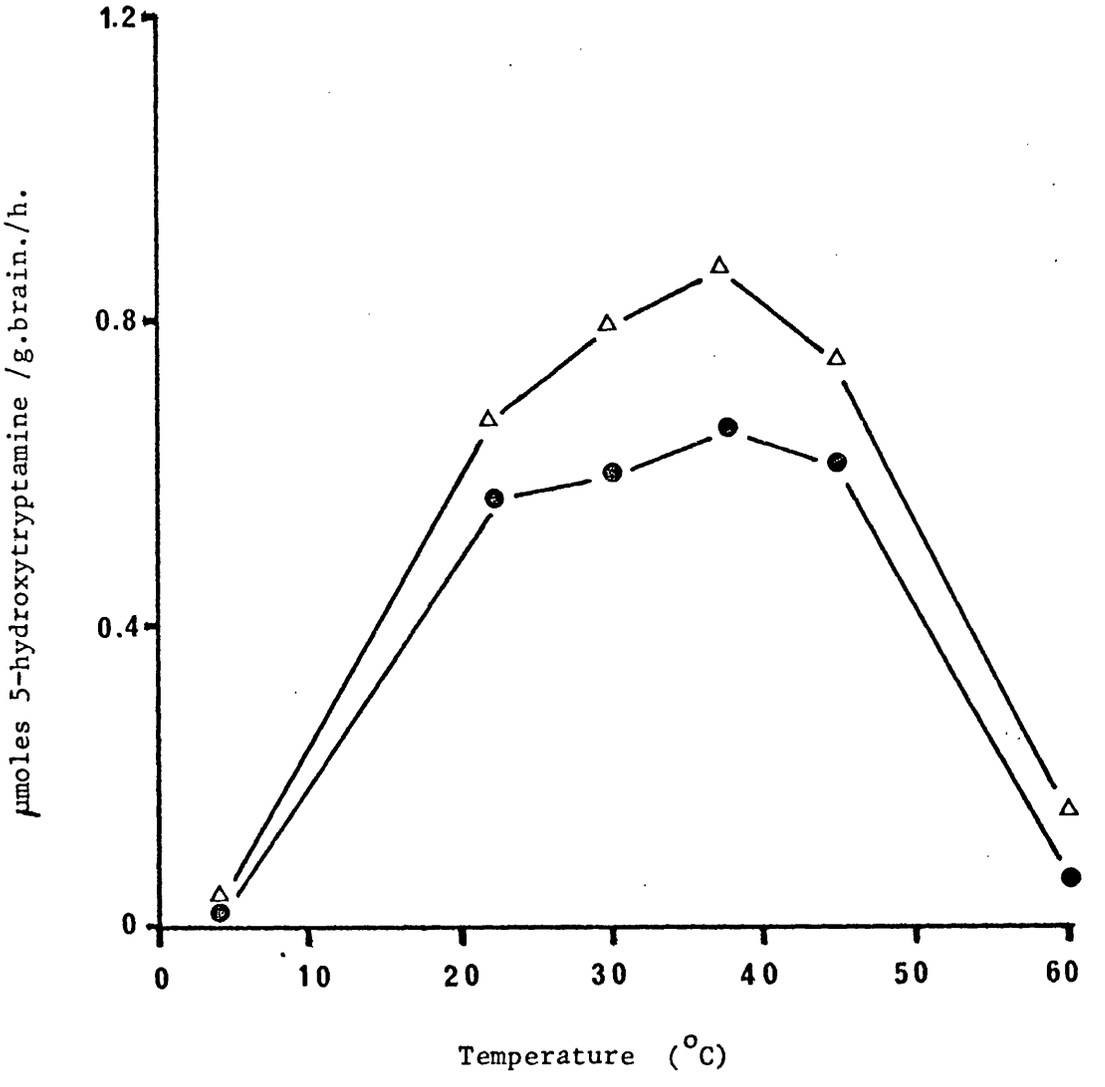


Figure 44. The effects of temperature on the activity of 5-hydroxytryptophan decarboxylase at 01.00 h (● —— ●) and 13.00 h. (△—— △) in a homogenate of whole brain. pH 8.0, incubation time 1h. 5-hydroxytryptophan 5 x 10^{-3}M. Results are the mean of three experiments.

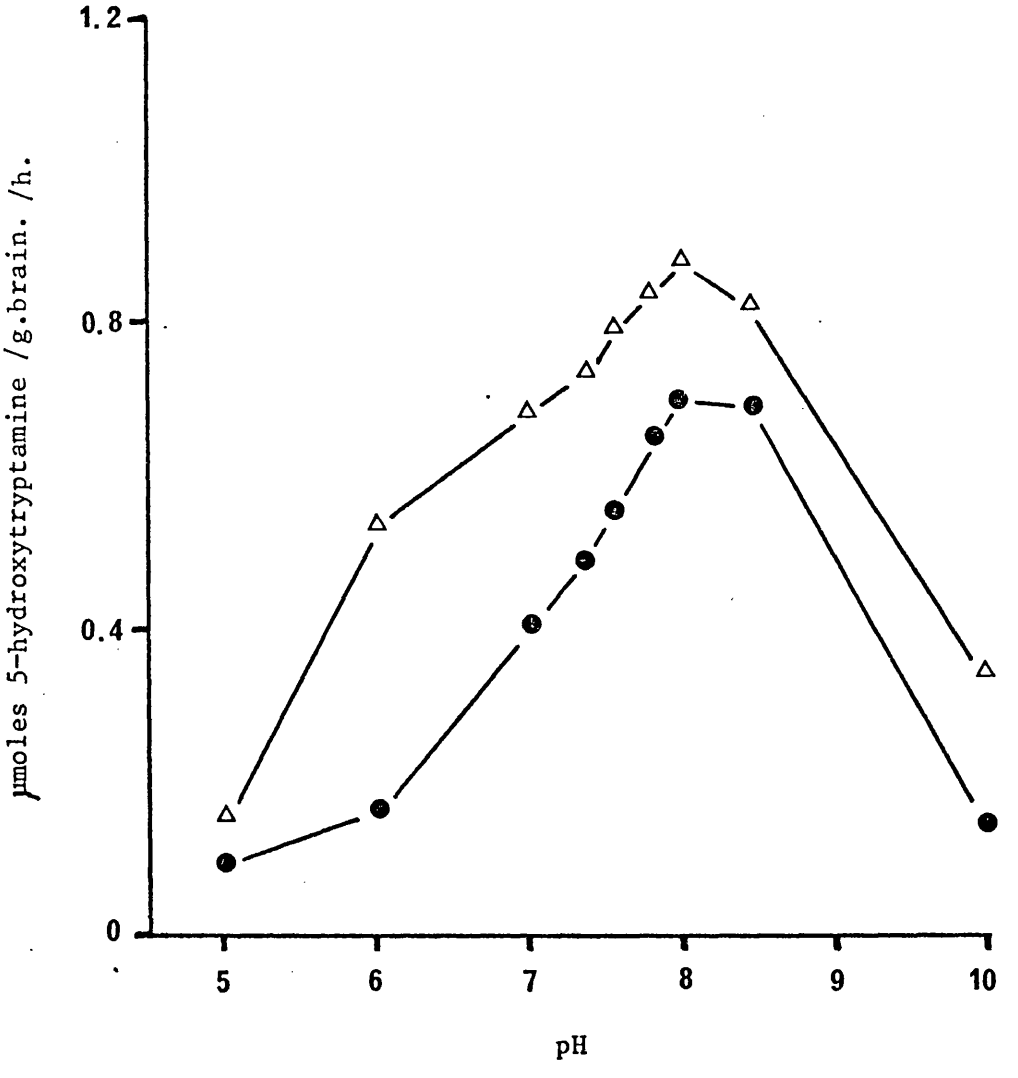


Figure 45. The effects of pH on the activity of 5-hydroxytryptophan decarboxylase at 01.00 h (●——●) and 13.00 h. (△——△) in a homogenate of whole brain. Incubated for 1h. at 37°C. 5-hydroxytryptophan 5 x 10^{-3}M. Results are the mean of three experiments.

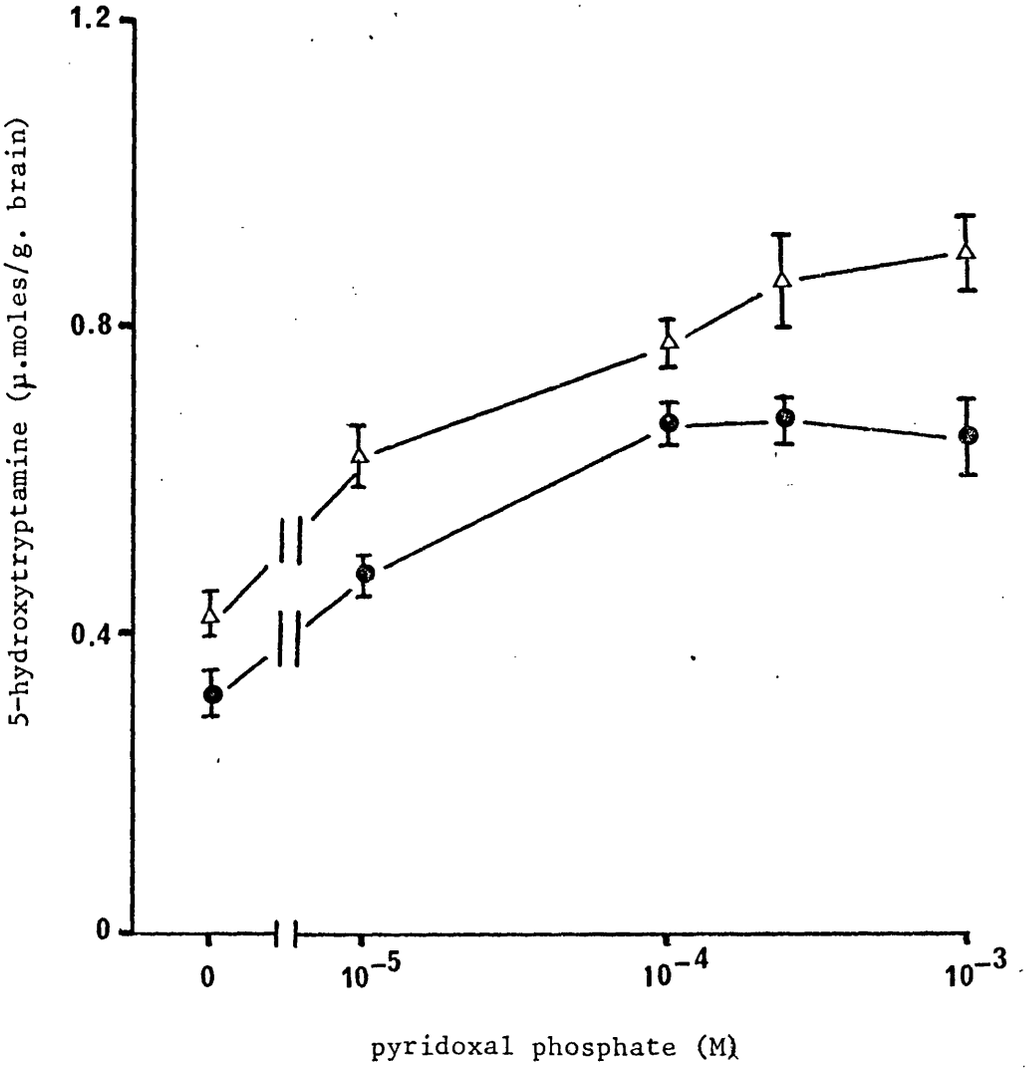


Figure 46. The effects of pyridoxal phosphate concentration on the activity of 5-hydroxytryptophan decarboxylase at 01.00 h (●——●) and 13.00 h. (Δ——Δ) in a homogenate of whole brain. Incubated for 1 h. at 37°C and pH 8.0. 5-hydroxytryptophan 5 x 10^{-3}M. Results are the mean of six experiments.

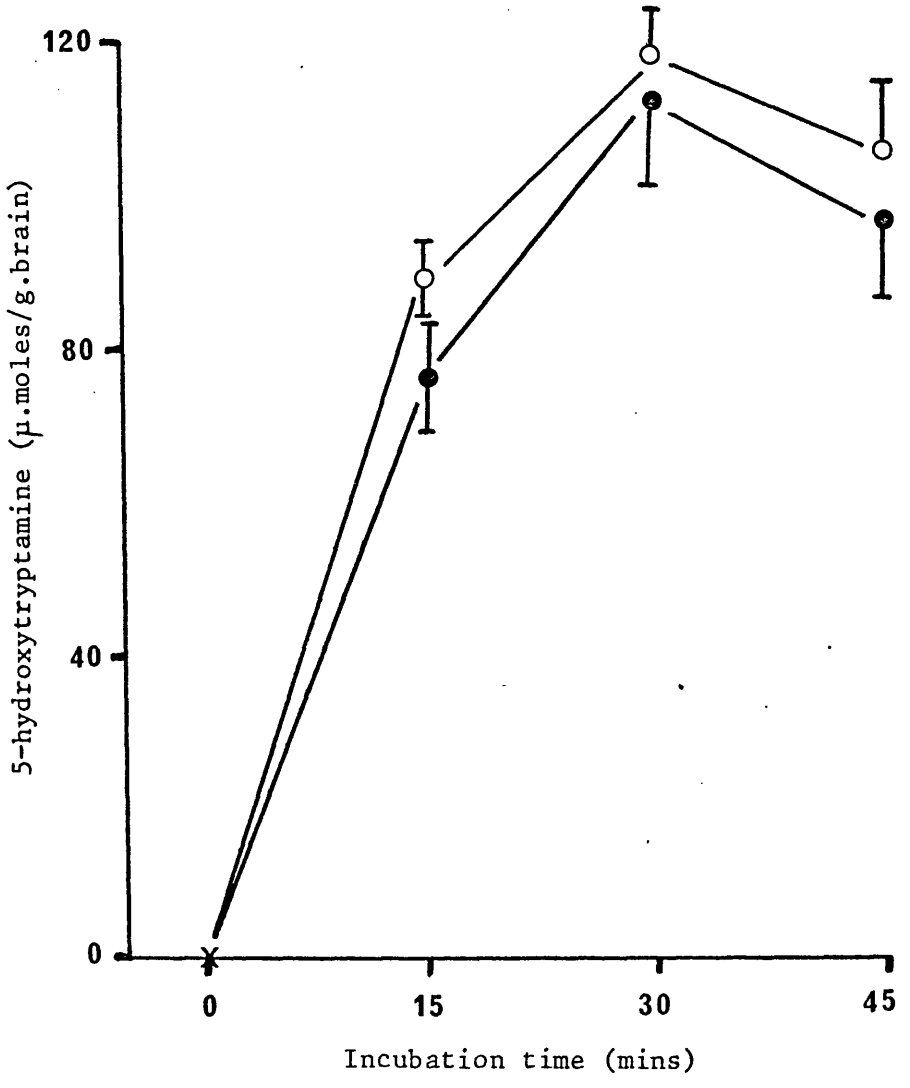


Figure 47. The activity of a purified preparation of 5-hydroxytryptophan decarboxylase at 01.00 h. (●——●) and 13.00 h. (○——○). Incubated at pH 8.0 for 1 h. at 37°C with 4 x 10^{-4}M pyridoxal phosphate. Results are the mean of ten experiments.

It was therefore concluded that there was no twenty-four hour variation in the activity of the enzyme.

Since no twenty-four hour variation of enzyme activity was found in a purified extract, and a twenty-four hour variation was shown in a crude homogenate it must be concluded that this variation in the activity of the enzyme is due to other factors. The addition of varying concentrations of pyridoxal phosphate did not cancel out the twenty-four hour variation so the availability of this co-factor does not appear to regulate the variation of enzyme activity. Equally, the availability of precursor did not appear to affect the activity of the enzyme since pretreatment with p-chlorophenylalanine did not significantly alter the rates of synthesis at 01.00 h. and 13.00 h. when measured in a crude homogenate (Figure 48). As a consequence of this last finding it appears that 5-hydroxytryptophan decarboxylase is probably unsaturated with its substrate.

The twenty-four hour variation of 5-hydroxytryptophan decarboxylase is therefore controlled by other factors, possibly by competition of the 5-hydroxytryptophan substrate with other amino acids e.g. DOPA.

As stated in Chapter Three of this thesis, maximal concentrations of 5-hydroxytryptamine and its precursors have been found at 01.00 h. and 13.00 h. It was therefore felt justified to take these two clock hours to determine many of the variables which might affect the concentration of 5-hydroxytryptamine in the rat brain. Unfortunately, in the case of 5-hydroxytryptophan decarboxylase it can now be seen (Figure 49) that highest activity was measured at 17.00 h. and lowest activity at 09.00 h. The twenty-four hour variation was not significantly different from the calculated sine curve (Figure 50), and the highest activity was significantly different from the lowest activity ($p < 0.001$) when compared by the Students t test. Also,

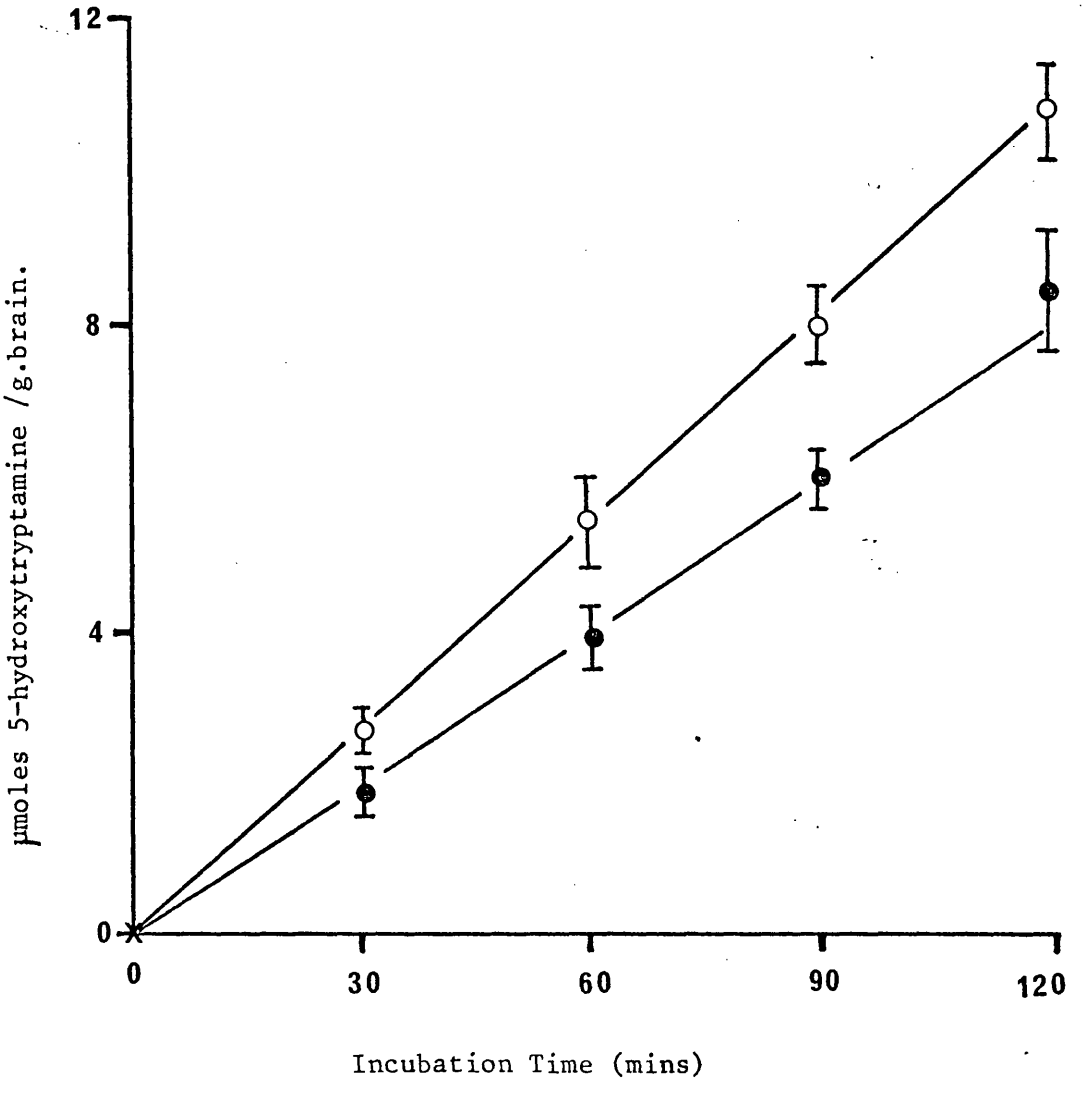


Figure 48. The activity of 5-hydroxytryptophan decarboxylase at 01.00 h. (● —— ●) and 13.00 h. (○ —— ○) following pretreatment of rats with p-CPA (316 mg/kg. p.o.). Whole brain homogenate incubated at pH 8.0 for 1 h. at 37°C. Results are the mean of eight experiments.

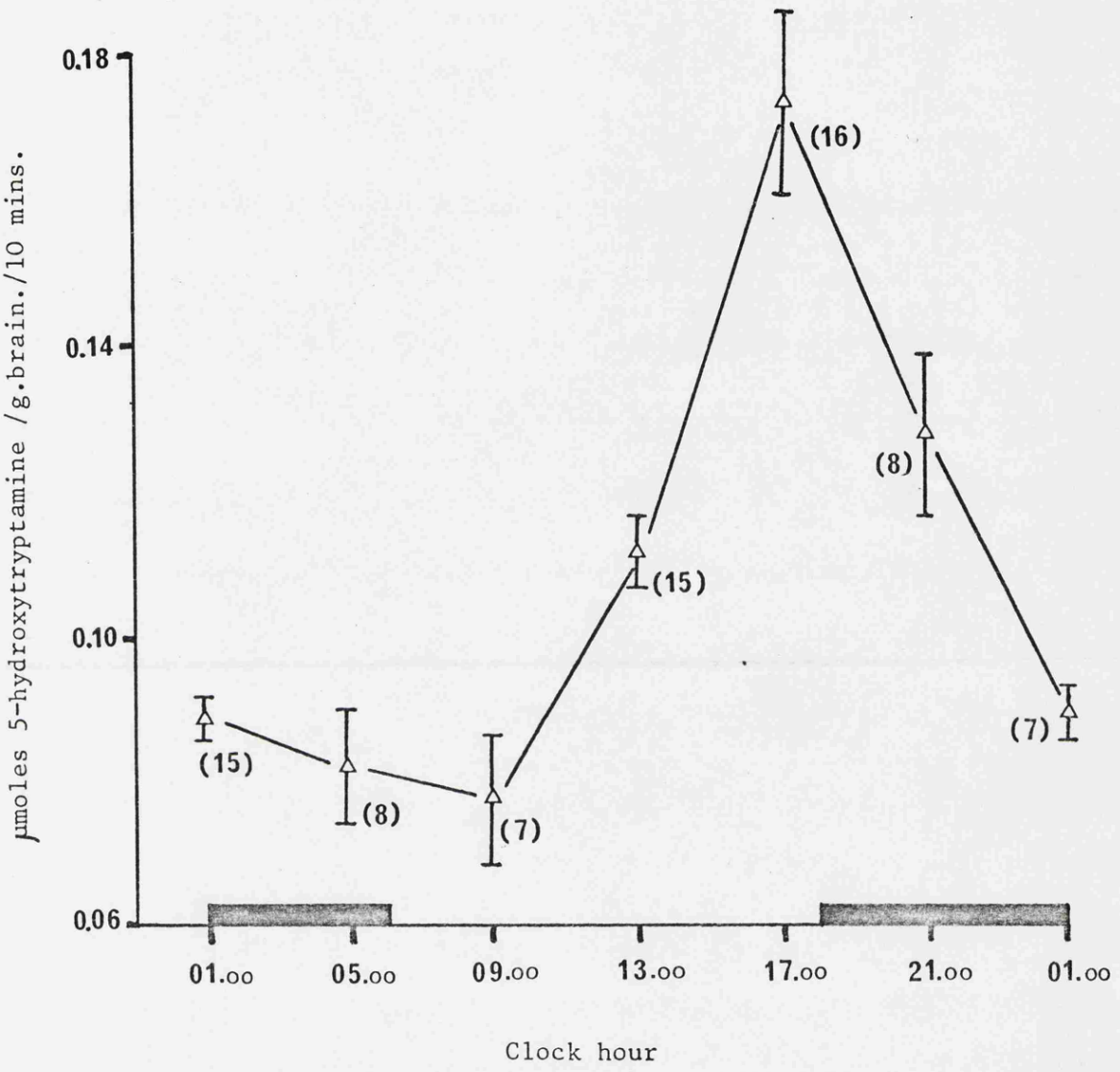


Figure 49. The twenty-four hour rhythm of 5-hydroxytryptophan decarboxylase activity (± SEM) in the rat brain. Solid black bars indicate the hours of darkness. The number of animals used are shown in parentheses.

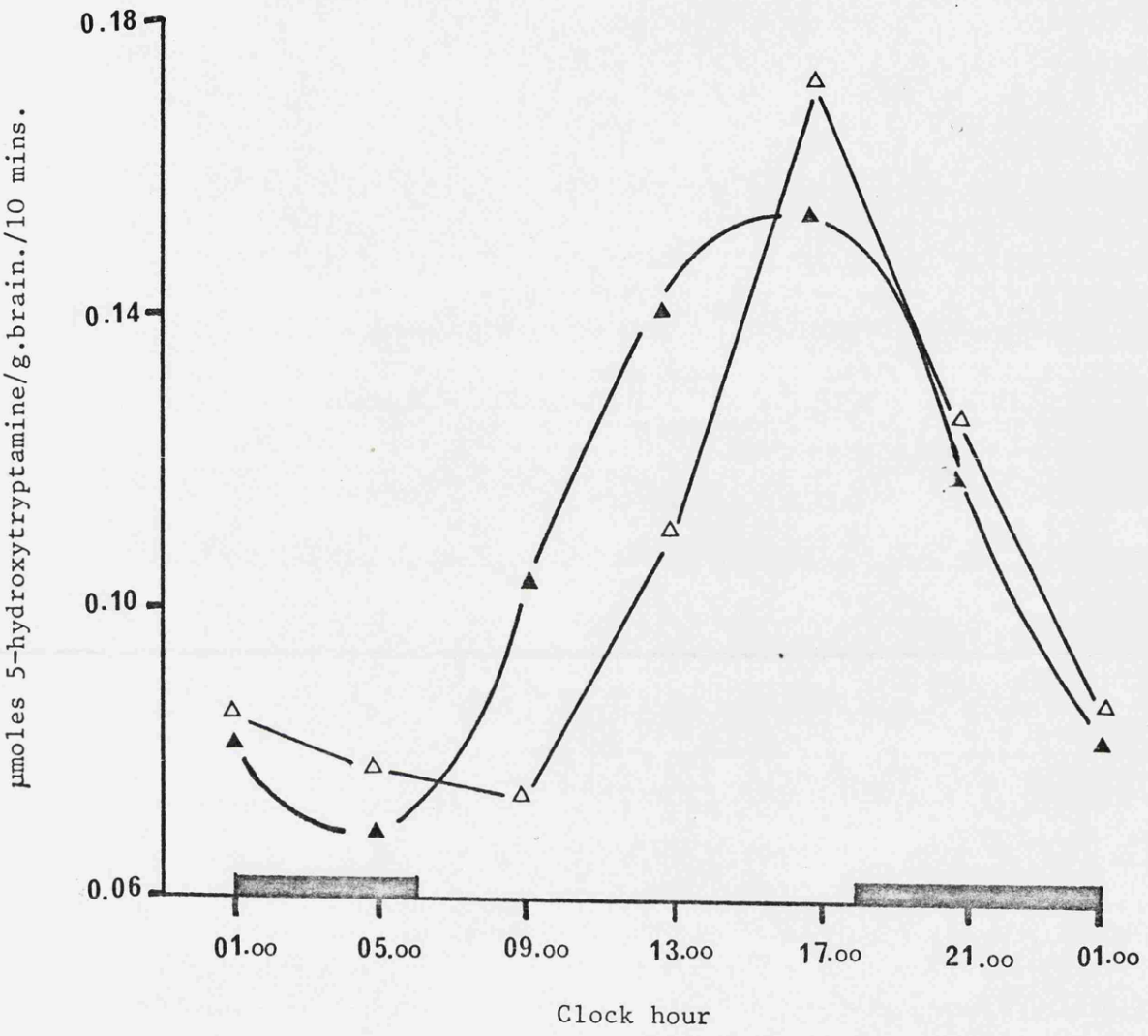


Figure 50. The twenty-four hour rhythm of 5-hydroxytryptophan decarboxylase activity compared with the calculated sine curve.

the activity at 13.00 h. was significantly different ($p < 0.001$) from the activity at 01.00 h.

The results obtained from these experiments indicate that 5-hydroxytryptophan decarboxylase activity or the availability of the enzyme to decarboxylate 5-hydroxytryptophan could play an important part in the production of the twenty-four hour rhythm of 5-hydroxytryptamine synthesis.

CHAPTER EIGHT

The activity of monoamine oxidase and the effects of monoamine oxidase inhibitors on the Twenty-four hour Rhythm of 5-hydroxytryptamine in the Rat Brain.

8.1 INTRODUCTION

Monoamine oxidase (EC.1.4.3.4) (MAO) has been widely reported to be important in the regulation of the effective concentrations of biologically active amines in the CNS (Davison, 1958; Page 1958; Axelrod 1959). All monoamines are susceptible to this enzyme and catecholamines and 5-hydroxytryptamine are therefore readily metabolised by MAO in neuronal tissues (Brodie, Spector and Shore 1959). MAO is an insoluble enzyme located mainly in the outer membrane of the mitochondria (Schnaitman, Erwin and Greenawalt 1967). Rodriguez and De Robertis (1962) reported 70.2% MAO activity in the mitochondrial fraction, 17.5% in the nuclear fraction and 12.5% in the microsomal fraction. The half life of the enzyme in the brain is approximately eleven days, and this half life bears no relation to the turnover rates of the monoamines (Goridis and Neff 1971). The activity of MAO is unaffected by raphe lesions which deplete 5-hydroxytryptamine concentrations (Marsden, Broch and Guldberg 1972).

Johnston (1968) suggested that there was more than one MAO in the brain. Gorkin (1969) came to a similar conclusion using liver extracts containing MAO. Huszti and Borsy (1968) suggested different forms of the enzyme inactivated 5-hydroxytryptamine and dimethoxyphenylethylamine.

Youdim, Collins and Sandler (1969) isolated four forms of MAO from rat brain, and also showed that harmaline and other β -carboline monoamine oxidase inhibitors (MAOI) increased 5-hydroxytryptamine concentrations in the rat brain without affecting noradrenaline and dopamine concentrations, thus providing further evidence for more than one enzyme, although the possibility exists that there are differing substrate specificities for different molecular forms of the same enzyme. Yang, Goridis and Neff (1972) agreed that there may

be multiple forms of MAO and also reported that some forms of the enzyme appear to be located in specific tissues.

The rate of deamination of 5-hydroxytryptamine is increased by L-DOPA (Karobath, Diaz and Huttunen 1972) and decreased by MAOI. MAOI are generally classified as hydrazine derivatives and non-hydrazine derivatives but have the common function of inhibiting the activity of MAO. The non hydrazine derivatives also increase the rate of conversion of 5-hydroxytryptophan to 5-hydroxytryptamine (Weber 1966). The characteristic behavioural pattern following the injection of MAOI plus tryptophan or 5-hydroxytryptophan (front paw clonus, piloerection, exophthalmia and body tremors) are related to the degree of MAO inhibition and not to 5-hydroxytryptamine concentrations (Weber 1966). The inhibition of MAO by pargyline is probably due to the MAOI combining irreversibly with the flavin co-factor of the enzyme, (Oreland, Kinemuchi and Yoo 1973) and it is possible that other MAOI act in a similar manner.

The specific activity of MAO reaches adult levels by two weeks post partum in all regions of the brain except in the cerebellum, and it is therefore apparent that all the components of the indoleamine pathway do not mature synchronously.

The twenty-four hour variation of MAO activity has not been widely investigated. Snyder, Axelrod and Zweig (1967) could not find such a variation in enzyme activity in the pineal gland. However, Hery, Rouer and Glowinski (1972) found a more pronounced oxidative deamination of newly synthetised 5-hydroxytryptamine in the hypothalamus and brain stem during darkness, but indicated that this was due to an increased release of the amine and not to changes in enzyme activity.

Since MAO catalyses the breakdown of all monoamines, changes in the ratios of enzyme concentrations following MAOI will alter animal behaviour. Valzelli and Garattini (1968) have summed up this situation.

"Considering that 5-hydroxytryptamine, noradrenaline and dopamine may exert an opposite or synergic effect on given receptors it is suggested that certain pharmacological effects of MAOI's may be associated not only with the increased level of brain amines, but also with new ratios which are established between the various amines in specific parts of the brain."

In this Chapter the activity of monoamine oxidase in the rat brain has been measured at two clock hours, and the effects of three monoamine oxidase inhibitors on the accumulation and synthesis rates of 5-hydroxytryptamine in the rat brain have been determined on a twenty-four hour basis.

8.2 METHODS

Male Sprague Dawley rats (120 - 140g) were maintained under the constant environmental conditions described in Chapter Two for ten days before each experiment.

8.2.1 Assay of Monoamine oxidase (MAO) (monoamine:O_2 oxidoreductase (deaminating) EC.1.4.3.4.) activity.

Acclimatised male Sprague Dawley rats, in groups of seven or eight, were killed by decapitation at either 01.00 h. or 13.00 h. The pineal gland was discarded and the brain rapidly removed, weighed and homogenised in ice-cold 0.25M sucrose (10 ml./g) and centrifuged twice at 1,500g. at $0^{o}C$ for ten minutes to precipitate excess debris. The supernatant was removed and centrifuged at 18,000g. at $0^{o}C$ for twenty minutes to obtain a crude mitochondrial fraction. The pellet was resuspended in 5 ml. 0.05M phosphate buffer pH 7.4 and centrifuged at 18,000g. at $0^{o}C$ for twenty minutes. The pellet was resuspended in 1.7 ml. 0.05M phosphate buffer and the activity of the MAO was determined as follows:

0.2 ml. DL-^{14}C-5-hydroxytryptamine (57 mCi/m.mole) (2×10^{-5}M) + 0.1 ml. non-radioactive DL-5-hydroxytryptamine (1×10^{-3}M) was added to the homogenate obtained above and was incubated at $37^{o}C$ for one hour in a shaking water bath. The reaction was stopped by boiling for five minutes at $100^{o}C$. ^{14}C-5-hydroxyindole-3-acetic acid was isolated by the chromatographic method described in Chapter Five, with the exception that only one-way paper chromatography was employed, using the propan-2-ol:ammonia:water solvent system. The areas containing the ^{14}C-5-hydroxyindole-3-acetic acid were cut out, eluted with 10 ml.water and evaporated to dryness under reduced pressure at $55^{o}C$, and made up

to 1.0 ml. with water. 0.5 ml. was added to 5 ml. Unisolve and taken for liquid scintillation counting. The rate of formation of 5-hydroxyindole-3-acetic acid was calculated from the results obtained.

8.2.2 The Effects of Monoamine Oxidase Inhibitors (MAOI) on the twenty-four hour rhythm of 5-hydroxytryptamine in the rat brain.

Male Sprague Dawley rats were maintained under constant environmental conditions for ten days before each experiment as described in Chapter Two. Rats were kept in groups of seven or eight.

In the first experiment, rats were injected intraperitoneally with pargyline (150 mg/kg) or saline (0.9%) and were killed by decapitation four hours later such that one group of pargyline treated and one group of saline treated was killed at 01.00 h. and one group of each was killed at 13.00 h. The concentration of 5-hydroxytryptamine in each group was determined by the method described in Chapter Three.

In the second experiment, rats were injected intraperitoneally with tranylcypromine (20 mg/kg) or saline (0.9%) and the procedure carried out as before using animals killed at 01.00 h. and 13.00 h.

In the third experiment rats were allowed free access to a solution of isocarboxazid (100 µg/ml.tap water), which replaced the normal drinking water, for either four or eight days. Drinking activity was monitored by the method described in Chapter Eleven. In the first instance groups of rats were killed, after four days,

at four-hourly intervals throughout twenty-four hours to determine the twenty-four hour variation of the effects of MAOI on 5-hydroxytryptamine concentrations. In the second instance groups of rats were killed after 4 days or 8 days at 01.00 h. and 13.00 h. only in order to determine the long term effects of MAOI on the twenty-four hour rhythm of 5-hydroxytryptamine concentrations in the rat brain.

8.3 RESULTS AND DISCUSSION

The rate of formation of 5-hydroxyindole-3-acetic acid from 5-hydroxytryptamine was 624 ± 11 mμ.moles/h^{-1} at 01.00 h. and 597 ± 13 mμ.moles/h^{-1} at 13.00 h. (Table 6). Each estimate was made as the rate formed per brain, since there was no significant difference between brain weights at these clock hours. There was no significant difference between the rates of 5-hydroxyindole-3-acetic acid formation at 01.00 h. and 13.00 h., and it was therefore concluded that there was no twenty-four hour variation of monoamine oxidase activity.

The concentration of 5-hydroxytryptamine in rats injected with saline (0.9%) were 249 ± 16 mμg/g^{-1} at 01.00 h. and 444 ± 11 mμg/g^{-1} at 13.00 h. (Table 7). These concentrations were significantly different at the $p < 0.001$ level by students t test. As expected there was therefore a twenty-four hour variation on 5-hydroxytryptamine concentration in control groups. Rats killed 4h. following the injection of pargyline (150 mg/kg i.p.) showed 5-hydroxytryptamine concentrations to be increased by 56% (388 ± 27 mμg/g^{-1}) at 01.00 h. and 87% (827 ± 40) at 13.00 h. These increases were statistically significant ($p < 0.001$) and the concentrations measured

Clock hour	n	mμ. moles 5-hydroxyindole-3-acetic acid formed/h/brain
01.00	14	624 ± 11
13.00	15	597 ± 13

Table 6. The activity of monoamine oxidase at 01.00 h and 13.00 h.

Treatment	5-hydroxytryptamine ($m\mu g/g^{-1}$)			
Clock hour	01.00	% increase	13.00	% increase
Saline (0.9%)	249 ± 16***	-	444 ± 11***	-
Pargyline (150 mg/kg)	388 ± 27***f	56	827 ± 40***f	87
Tranylcypromine (20 mg/kg)	469 ± 36***ff	89	921 ± 27***ff	108

Table 7. The synthesis of 5-hydroxytryptamine at 01.00 h. and 13.00 h. following inhibition of monoamine oxidase with pargyline and tranylcypromine. Animals were killed after four hours.

*** $p < 0.001$ 01.00h. compared with 13.00 h.

f $p < 0.001$ pargyline compared with control

ff $p < 0.001$ tranylcypromine compared with control.

at 01.00 h. were significantly different from those at 13.00 h. ($p < 0.001$).

5-hydroxytryptamine concentrations in animals injected with tranylcypromine (20 mg/kg i.p.) were also increased when compared with control values; 89% (469 $\pm$ 36 $m\mu g/g^{-1}$) at 01.00 h. and 108% (921 $\pm$ 27) at 13.00 h. These values were significantly different from control values ($p < 0.001$) and the values measured at 01.00 h. were significantly different ($p < 0.001$) from values measured at 13.00 h.

The net increase in 5-hydroxytryptamine concentrations was greater at 13.00 h. than at 01.00 h. in both pargyline-treated and tranylcypromine-treated rats, and the synthesis rate of the amine was therefore greater at 13.00 h. when compared with 01.00 h. It is thus apparent that there is a twenty-four hour variation in the rates of synthesis of 5-hydroxytryptamine when measured in vivo by this method of MAO inhibition, with the higher rate at 13.00 h. and the lower rate at 01.00 h.

Isocarboxazid administered in the drinking water (100 $\mu g/ml$). increased 5-hydroxytryptamine concentrations in the rat brain throughout the twenty-four hours (Figure 51). Water consumption was approximately 25 ml./rat/day with 95% of the drug being consumed during the dark period. Each rat therefore consumed approximately 2.5 mg. isocarboxazid per day. There was no significant variation in 5-hydroxytryptamine concentrations with clock hour in isocarboxazid-treated rats (Figure 51). Concentrations of the amine were therefore stable throughout the twenty-four hours, and this must presumably be due to the synthesis of 5-hydroxytryptamine being greater than the rate of release and degradation of the amine.

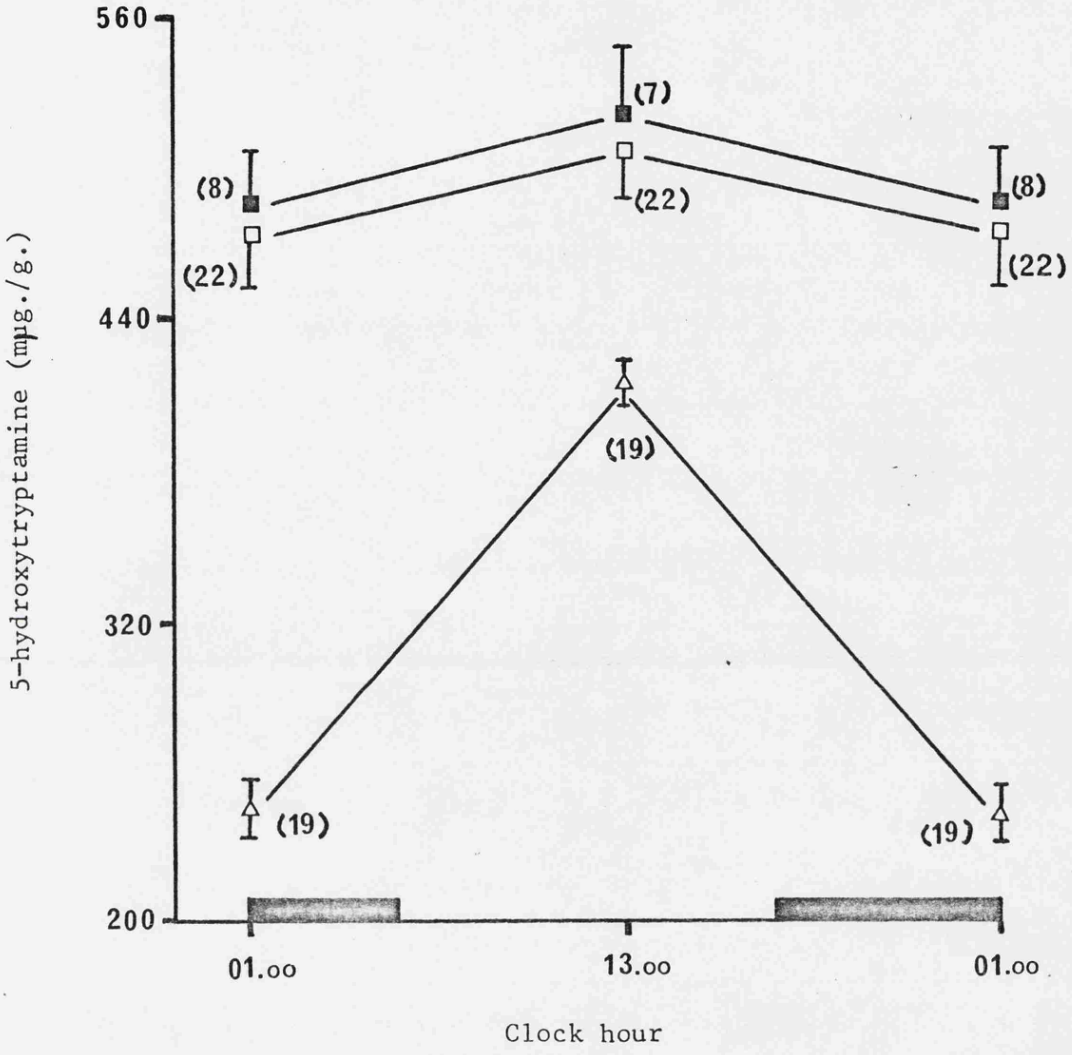


Figure 51. The effects of isocarboxazid (□—□ 4 days) (■——■ 8 days) (△——△ control) administered in the drinking water (100 μg/ml) on the twenty-four hour variation of 5-hydroxytryptamine concentrations (± SEM) in the rat brain (measured at 01.00 h. and 13.00 h. only). Numbers of animals used are shown in parentheses.

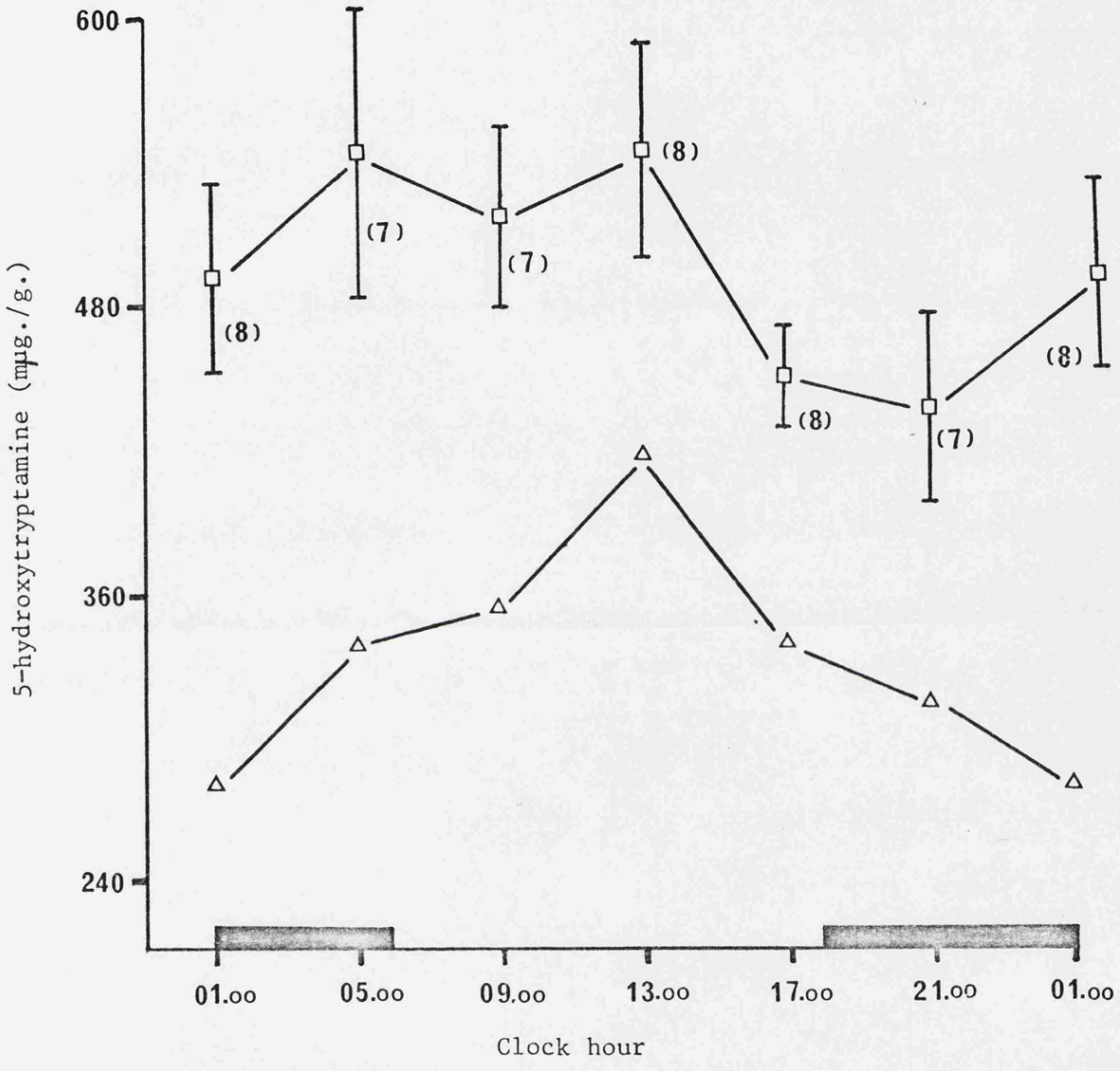


Figure 52. The effects of isocarboxazid (100 μg/ml. in the drinking water for 4 days) (□——□) on the twenty-four hour variation of 5-hydroxytryptamine concentrations (± SEM) in the rat brain. Control (△——△). The number of animals used are shown in parentheses.

There was no significant difference in 5-hydroxytryptamine concentrations between rats receiving isocarboxazid for four days and for eight days (Figure 52). A maximum concentration of the amine therefore appears to have been reached, possibly by feedback mechanisms inhibiting the synthesis of the amine, thus preventing further increases of 5-hydroxytryptamine concentrations in the rat brain.

The twenty-four hour rhythm of 5-hydroxytryptamine concentrations was abolished by the use of isocarboxazid in the drinking water after four and eight days, it was therefore apparent that the presence of monoamine oxidase is essential to maintain the rhythm of 5-hydroxy-tryptamine concentrations in the rat brain. The mechanism by which monoamine oxidase exerts this controlling influence must be indirect since the activity of the enzyme itself did not vary with clock hour. It must therefore depend on the availability of substrate to exert an effect. The availability of substrate for degradation is dependent upon the activity of 5-hydroxytryptamine-containing neurones, and the activity of these neurones can be determined by the measurement of 5-hydroxyindole-3-acetic acid concentrations in the rat brain (5-hydroxyindole-3-acetic acid concentrations have been determined over a twenty-four hour period, and the results are presented in the following Chapter).

CHAPTER NINE

The twenty-four hour rhythm of 5-hydroxyindole-3-acetic acid concentrations in the rat brain

9.1 INTRODUCTION

5-hydroxyindole-3-acetic acid is formed from 5-hydroxytryptamine by oxidative deamination involving monoamine oxidase and aldehyde dehydrogenase. The acid has been identified in the cerebrospinal fluid (CSF) of the dog (Twarog and Page 1953) and the rat (Ashcroft and Sharman 1960) and in the brain of the rat (Sharman 1960). The acid does not easily enter the brain (Moir and Eccleston 1968) but concentrations rapidly increase following injection of tryptophan (Schubert 1974) or 5-hydroxytryptophan (Roos 1962) or following stimulation of the raphe nuclei. (Eccleston, Padjen and Randic 1969). The distribution of 5-hydroxyindole-3-acetic acid in the brain is the same as the distribution of 5-hydroxytryptamine and the acid is therefore probably formed at the same site in the tissue (Roos 1962). Indeed the concentration of 5-hydroxyindole-3-acetic acid in the CSF reflects the metabolism of 5-hydroxytryptamine in the brain (Bowers 1970; Roos, Anden and Werdinius 1964; Moir, Ashcroft, Crawford, Eccleston and Guldberg 1970), and similarly changes in the brain concentration of the acid reflect the turnover of brain 5-hydroxy-tryptamine (Perez-Cruet, Tagliamonte, Tagliamonte and Gessa 1972, Bliss, Thatcher and Ailion 1972). On the other hand changes in the concentration of the acid itself do not influence the turnover of the amine (Neff and Tozer 1968).

5-hydroxyindole-3-acetic acid is the major excretory product of 5-hydroxytryptamine metabolism (Chapter One) and must therefore be removed from the brain to the circulation. The efflux of the acid from the brain has components due to diffusion, a saturable transport mechanism and bulk flow (Ashcroft, Dow and Moir 1968; Sampath and Neff (1974). The transport mechanism is apparently situated in the

choroid plexuses of the fourth ventricle although only 10% of the total 5-hydroxyindole-3-acetic acid is removed through the CSF (Meek and Neff 1973). Evidence for the use of bulk flow to remove metabolites has been presented by Guldberg, Ashcroft and Crawford (1966). These workers demonstrated a marked gradient of 5-hydroxyindole-3-acetic acid concentrations from the lateral ventricles to the cisterna magna of the dog. However, Sampath and Neff (1974) concluded that most of the acid found in the brain is transported directly into the circulation and only a small fraction enters the CSF. The choroid plexuses were not the only sites of acid transport in the brain.

Nearly all studies to determine rates of metabolism of 5-hydroxytryptamine by measuring 5-hydroxyindole-3-acetic acid concentrations have employed the drug probenecid which inhibits the removal of the acid from the CNS (Korf, Van Praag and Sebens 1971). Since the rate of accumulation of 5-hydroxyindole-3-acetic acid following the injection of probenecid is almost identical with the rate of formation of 5-hydroxytryptamine it has been implied that 5-hydroxyindole-3-acetic acid is transferred directly from brain tissue to the plasma under normal conditions (Neff, Tozer and Brodie 1967).

The twenty-four hour variation of 5-hydroxyindole-3-acetic acid concentrations has not been extensively investigated. However, Morgan, Yndo and Mcfadin (1974) found significant daily changes in the concentrations of the acid in the brain of mice, although these daily changes did not correlate with the concentrations of 5-hydroxytryptamine that were measured. Quay (1964 (a)) found a twenty-four hour variation of 5-hydroxyindole-3-acetic acid concentrations in the pineal glands of female rats and this variation, which generally resembled that of 5-hydroxytryptamine in the pineal gland, had the following characteristics:

The time of maximum content of the acid was significantly later than that of the amine. The drop to a nocturnal minimum was more rapid. The nocturnal basal level was maintained for at least five hours before the start of the subsequent morning rise. As a result of these findings, Quay (1964(a)) concluded that the later rise and the maximum point of 5-hydroxyindole-3-acetic acid concentrations in the 5-hydroxyindole-3-acetic acid rhythm when compared with the 5-hydroxytryptamine rhythm could indicate that the former is derived from the latter.

In this Chapter the twenty-four hour variation of 5-hydroxyindole-3-acetic acid concentrations has been determined.

9.2 METHODS

Male Sprague Dawley rats (120 -140g) were maintained under the constant environmental conditions described in Chapter Two for ten days before each experiment.

9.2.1 Assay of 5-hydroxyindole-3-acetic acid (Giacalone and Valzelli 1966)

Rats were killed by decapitation, the pineal glands discarded and the brains immediately removed, weighed and homogenised in 3.0 ml. 0.1N HCl containing 0.5% L-ascorbic acid,using a glass tube and teflon pestle as described previously. The homogenate was deproteinised using 0.5 ml. 10% (w/v) $ZnSO_4.7H_2O$ and 0.3 ml. N/1. NaOH. The mixture was centrifuged at 4,000 r.p.m. for ten minutes in a bench centrifuge. The supernatant was filtered through glass wool and 0.5 ml. 2% (w/v) EDTA, 0.5 ml. 6% (w/v) L-ascorbic acid and 0.65 ml. N/1. HCl were added to the filtrate. The homogeniser tube and pestle were washed with 3.0 ml. 0.1N. HCl containing 0.5% (w/v) L-ascorbic acid. The washings were added to the precipitate obtained previously and the precipitate was resuspended. 0.5 ml. 10% (w/v) $ZnSO_4$ $7H_2O$ and 0.3 ml. N/1. NaOH were added and the mixture was centrifuged at 4000 r.p.m. in a bench centrifuge. The supernatant was filtered through glass wool and the filtrate was added to the first filtrate obtained. 3.0g. NaCl and 5 ml. butyl acetate were added to the combined filtrates, the mixture was shaken for five minutes, and centrifuged for two minutes at 5000 g. 4.5 ml. organic phase was added to 4.0 ml. 0.1N. HCl containing 0.5% (w/v) L-ascorbic acid saturated with NaCl. The mixture was shaken for five

minutes and centrifuged for two minutes at 5000g. 4.0 ml. organic phase was added to 1.3 ml. 0.1M. phosphate buffer pH 7.0 containing 0.1% (W/v) L-ascorbic acid. The mixture was shaken for five minutes and centrifuged for two minutes at 5000g. 1.0 ml. aqueous phase was made 3N with concentrated HCl and the fluorescence of the product was measured at excitation wavelength 295 mμ, emission wavelength 540 mμ (wavelengths uncorrected) in an Aminco-Bowman spectrophotofluorometer. The fluorescence spectrum of the isolated product was compared with pure 5-hydroxyindole-3-acetic acid (Sigma) to validate the assay procedure.

Internal and external standards and phosphate buffer blanks were measured during each assay in order to calculate a more accurate estimate of 5-hydroxyindole-3-acetic acid concentrations in the rat brain.

9.2.2 The Twenty-four hour variation of 5-hydroxyindole-3-acetic acid concentrations in the rat brain.

Rats were killed in groups of seven or eight at four-hourly intervals commencing at 09.00 h. and 5-hydroxyindole-3-acetic acid was assayed as described, in order to measure the twenty-four hour variation of 5-hydroxyindole-3-acetic acid in the rat brain. The significance of the variation was determined by Fourier analysis and the X^2 test.

9.3. RESULTS AND DISCUSSION

The fluorescence spectrum for the isolated product was identical with that of pure 5-hydroxyindole-3-acetic acid (Sigma) (Figure 53). When tryptophan, 5-hydroxytryptophan and 5-hydroxyindole-3-acetic acid were carried through the extraction and assay procedure they did not produce fluorescence greater than that observed with a phosphate buffer blank. The product measured in the assay was therefore assumed to be 5-hydroxyindole-3-acetic acid.

A twenty-four hour rhythm of 5-hydroxyindole-3-acetic acid concentrations has been shown (Figure 54) with highest concentrations (644.2 ± 10.4 $m\mu g/g^{-1}$) at 17.00 h. and lowest concentrations (426.9 ± 19.5 $m\mu g/g^{-1}$) at 01.00 h. Highest concentrations were therefore reached four hours after those of 5-hydroxytryptamine although this peak still occurred during the light period. Concentrations of the acid decreased rapidly during the early hours of darkness to reach a minimum at 01.00 h. Although the hour of lowest concentrations was the same when acid and amine were compared, the rate of decline was much greater for the acid.

The twenty-four hour variation of 5-hydroxyindole-3-acetic acid concentration was not significantly different from the calculated sine curve (Figure 55) and highest concentrations were significantly different from lowest concentrations when compared by the Students' t test ($p < 0.001$).

Since it has already been shown (Chapter Eight) that the activity of monoamine oxidase did not vary with clock hour, it was concluded that the concentrations of 5-hydroxyindole-3-acetic acid measured were a consequence of the amounts of 5-hydroxytryptamine released from 5-hydroxytryptamine-containing neurones and broken down by monoamine oxidase.

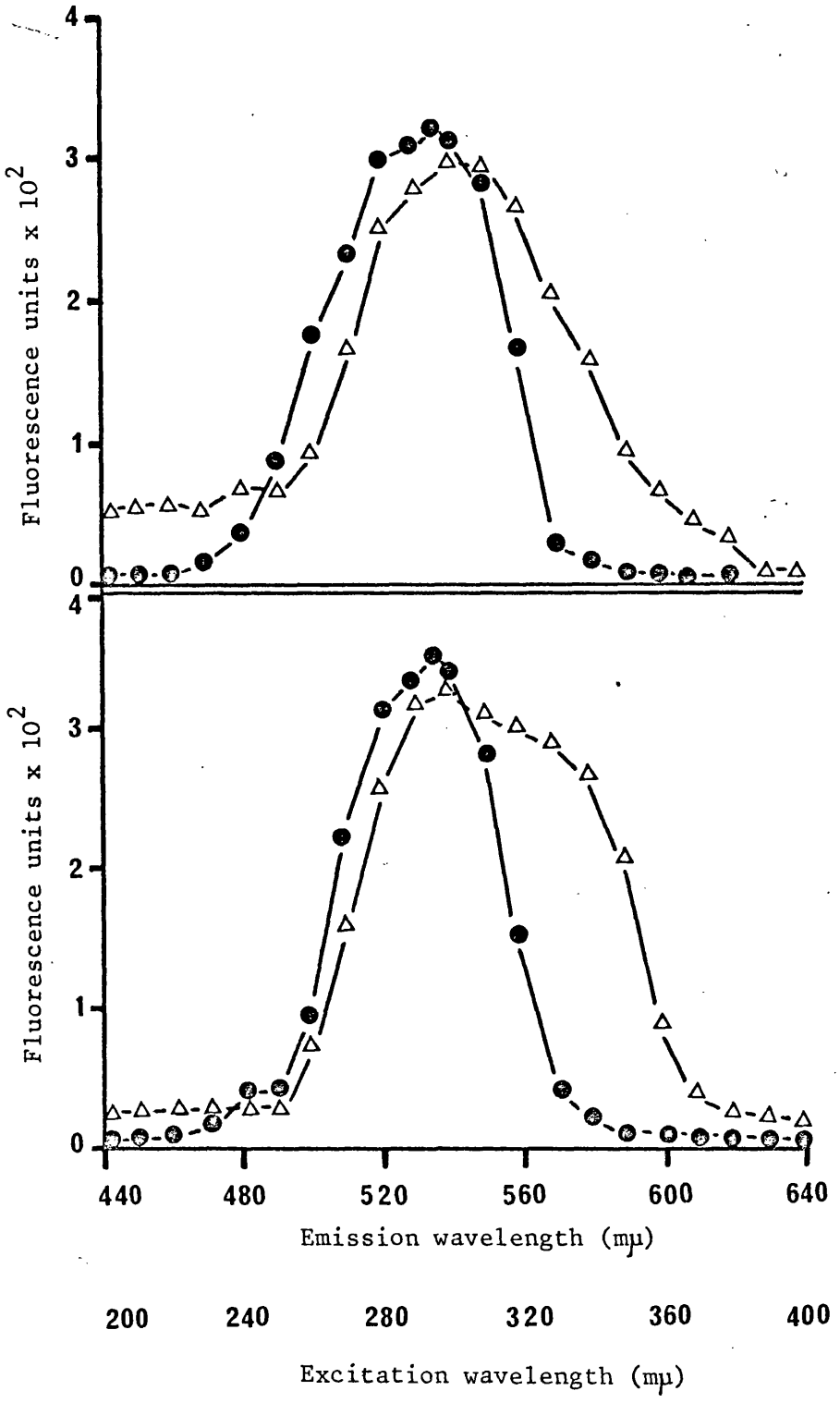


Figure 53. The Fluorescence spectrum of extracted 5-hydroxyindole-3-acetic acid (a) compared with that of the pure compound (b): (△——△). Excitation 295 mμ, scan emission; (●——●) Emission 540 mμ, scan excitation.

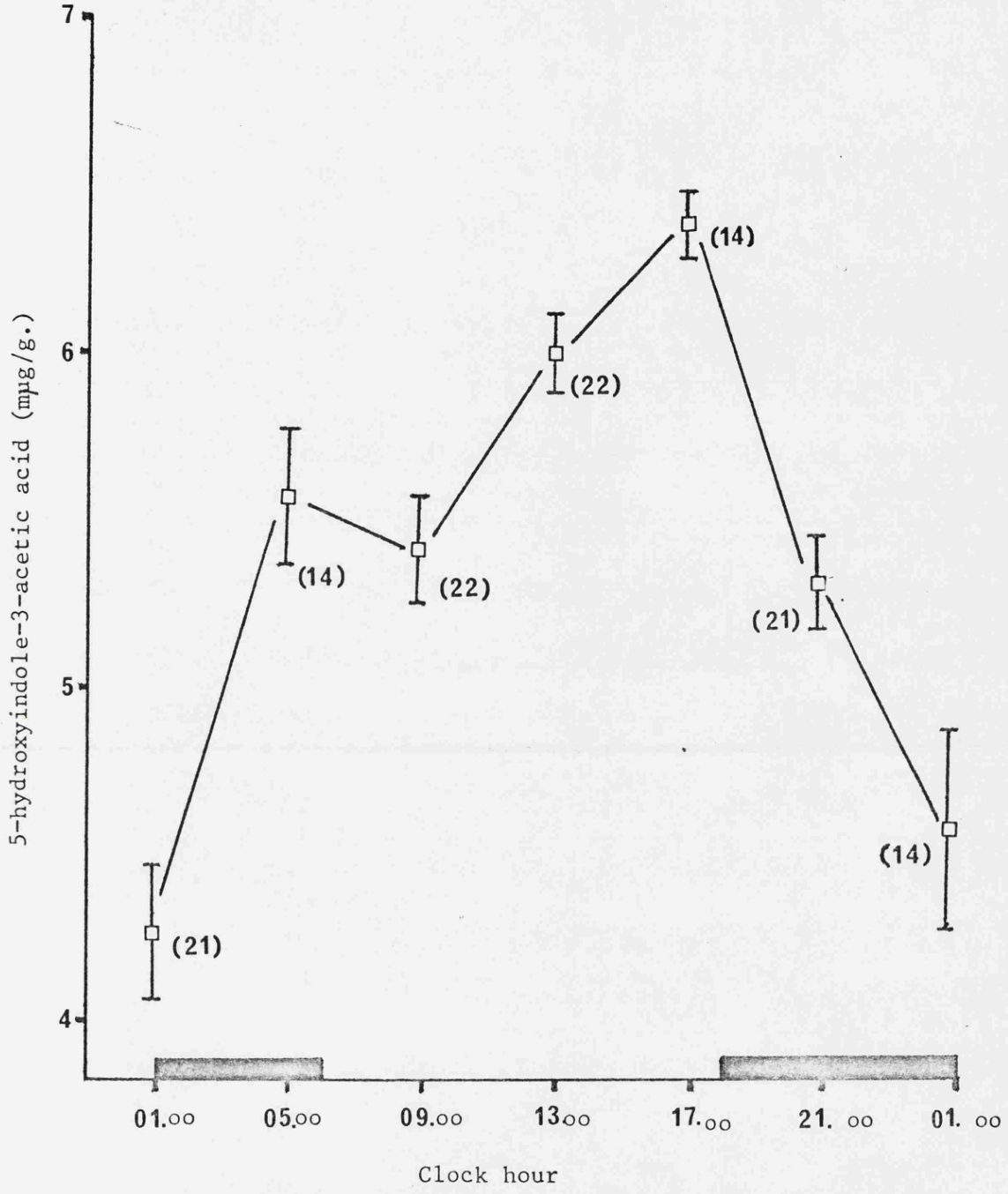


Figure 54. The twenty-four hour variation of 5-hydroxyindole-3- acetic acid concentrations (± SEM) in the rat brain.

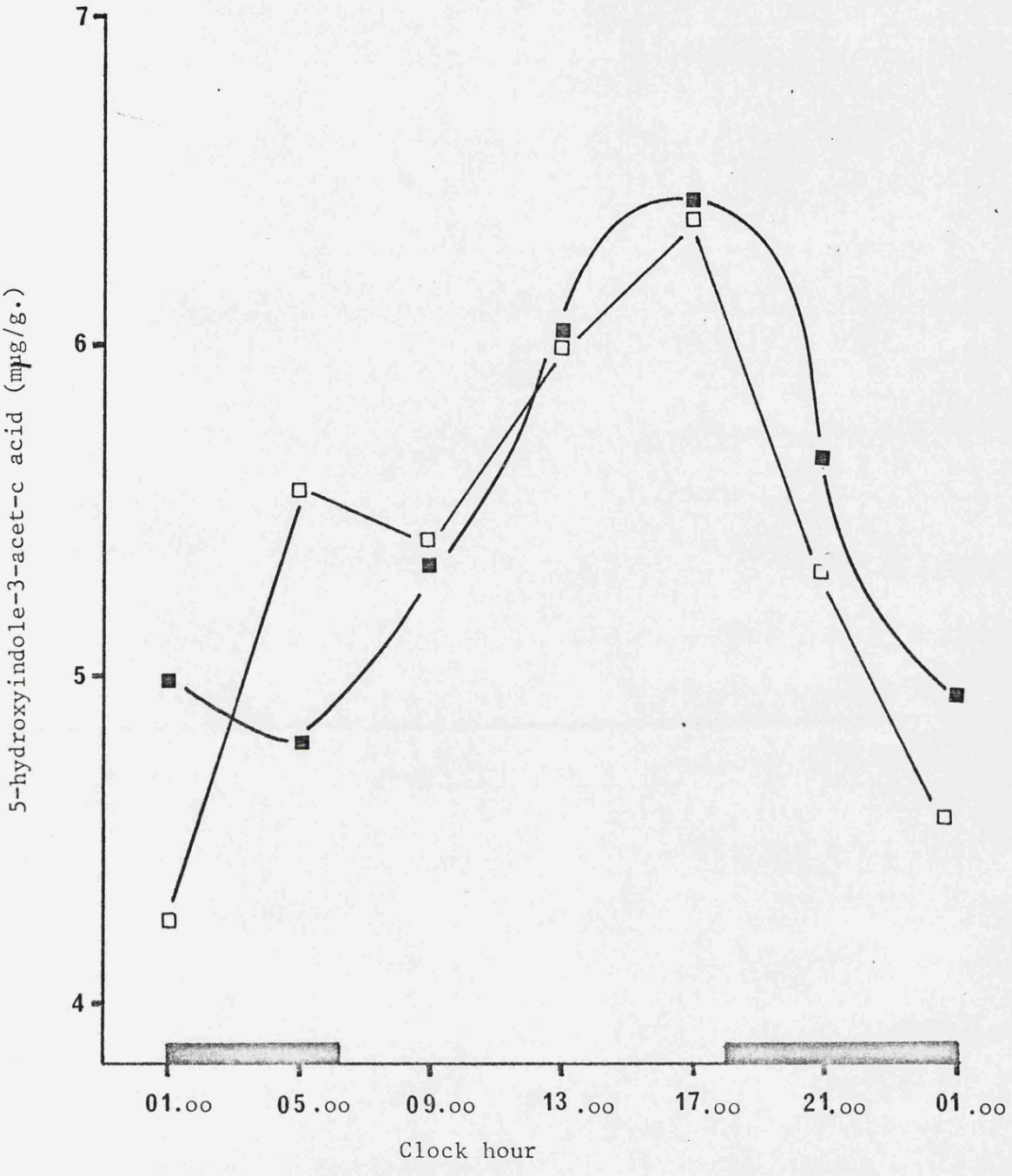


Figure 55. The twenty-four hour variation of 5-hydroxyindole-3- acetic acid concentrations in the rat brain compared with the calculated sine curve.

5-hydroxyindole-3-acetic acid concentrations were therefore considered to be a reflection of the activity of 5-hydroxytryptamine-containing neurones. The activity of these neurones was therefore highest during the light period and lowest during the dark period.

It has been suggested (Hery, Rouer and Glowinski 1972) that the twenty-four hour rhythm of 5-hydroxytryptamine concentrations may be controlled in part by an increased release of the amine during the dark period. This has clearly not been found to be the case since the concentrations of 5-hydroxyindole-3-acetic acid were lowest during this period.

So far in this thesis the synthesis and breakdown mechanisms relating to 5-hydroxytryptamine have been investigated in an attempt to identify controlling factors in the production of the twenty-four hour rhythm of 5-hydroxytryptamine. On several occasions catecholamines have been implicated in the production of this rhythm. The effects of catecholamine synthesis inhibitors on the twenty-four hour rhythm of 5-hydroxytryptamine were therefore investigated and the results are presented in the following Chapter.

CHAPTER TEN

The Effects of Inhibitors of catecholamine synthesis on the twenty-four hour variation of 5-hydroxytryptamine concentrations in the rat brain.

10.1 INTRODUCTION

The concentrations of noradrenaline in the brain of rats and cats exhibit a twenty-four hour or circadian rhythm with highest concentrations normally found during the dark period (Graziani and Montanaro 1967; Friedman and Walker 1968; Manshardt and Wurtman 1968; Reis and Wurtman 1968; Davies 1971). The rhythm of noradrenaline concentrations is therefore opposite in phase to the rhythm of 5-hydroxytryptamine concentrations.

Scheving, Harrison, Gordon and Pauly (1968) were unable to detect a rhythm of either dopamine or noradrenaline concentrations in the rat brain, and suggested that catecholamines are characterised by reproducible "higher frequency ultradian" rhythms. However previous work in our own laboratories (Davies 1971) has shown a rhythm of dopamine concentrations with two peaks (08.00 h and 1600 h) and therefore three periods of low concentrations (00.00 h, 12.00 h and 20.00 h). Concentrations of dopamine were therefore generally higher during the light period and approximately in phase with 5-hydroxytryptamine concentrations.

Reis, Weinbren and Corvelli (1968) and Reis, Corvelli and Conners (1969) found that the noradrenaline rhythm was endogenous or free running, and that the 5-hydroxytryptamine rhythm was independent of the noradrenaline rhythm in the brain regions of the cat that were studied.

If all these results are considered together it becomes apparent that, unlike the rhythm of 5-hydroxytryptamine concentrations, the twenty-four hour rhythms of catecholamine concentrations vary according to brain region. As a result, when whole brain, or parts of brain containing more than one brain region are taken for assay, ultradian rhythms may be seen. However noradrenaline rhythms normally show highest concentrations during the dark period.

It is worth reiterating at this stage that some evidence is available that catecholamines can play a part in controlling 5-hydroxytryptamine concentrations in the brain of the rat. Thus the concentrations of 5-hydroxytryptamine in the pineal gland are regulated by sympathetic innervation (Zweig and Azelrod 1969), and the maturation of the twenty-four hour rhythm of 5-hydroxytryptamine in the brain occurs only following the maturation of noradrenaline concentrations (Asano 1971).

The effects of inhibitors of catecholamine synthesis or destruction of catecholamine-containing neurones on the twenty-four hour rhythm of 5-hydroxytryptamine concentrations have not been widely investigated. However Hery, Rouer and Glowinski (1973) investigated the effects of 6-hydroxydopamine(6-OHDA) on the rhythm in the rat hypothalamus and found that the decrease in 5-hydroxytryptamine concentrations associated with the dark period did not occur following the intraventricular injection of 6-OHDA. These workers concluded that the diurnal variation in 5-hydroxytryptamine synthesis seemed to be controlled in part by central catecholamine-containing neurones since it was unlikely that 6-OHDA was toxic to 5-hydroxytryptamine-containing neurones. Noradrenaline concentrations were markedly decreased when 5-hydroxytryptamine concentrations were estimated three weeks after 6-OHDA injection.

In this Chapter I have investigated the effects of a tyrosine hydroxylase inhibitor (α -methyl-p-tyrosine) (Levitt, Spector, Sjoerdsma and Udenfriend 1965), a dopamine-β-hydroxylase inhibitor (FLA-63) (Anden and Fuxe 1971), and the effects of 6-OHDA on the twenty-four hour rhythm of 5-hydroxytryptamine concentrations in the rat brain.

It was intended that such an experimental design would allow the effects of noradrenaline and dopamine on the rhythm to be determined separately. The possible interactions between catecholamines and 5-hydroxytryptamine are discussed.

10.2 METHODS

Male Sprague Dawley rats (120 - 140g) were maintained under the constant environmental conditions described in Chapter Two for ten days before each experiment.

10.2.1 The Effects of FLA-63 (bis(4-methyl-1-homopiperazinylthiocarbonyl) disulphide) on the twenty-four hour variation of 5-hydroxytryptamine concentrations in the rat brain.

Two groups of seven rats were injected intraperitoneally with FLA-63 (25 mg/kg. made up in N/1.HCl), and a further two groups of seven rats were injected intraperitoneally with N/1.HCl. These latter groups served as controls. The four groups were killed by decapitation, three and a half hours after injection so that one group of FLA-63 treated and one group of controls were killed at 01.00 h., and the remaining groups were killed at 13.00 h. The pineal glands were discarded and the brains were immediately removed, frozen in liquid nitrogen, and stored at $-14^{o}C$. 5-hydroxytryptamine was assayed by the method of Snyder, Axelrod and Zweig (1965) as described in Chapter Three.

The experiment was repeated exactly as described except that FLA-63 (25 mg/kg.) was injected orally.

10.2.2 The Effects of α-methyl-p-tyrosine (α-MPT) on the twenty-four hour variation of 5-hydroxytryptamine concentrations in the rat brain.

Two groups of ten rats were injected intraperitoneally with α-MPT-methyl ester (250 mg/kg. 2ml/kg.). A further two groups of ten rats were injected intraperitoneally with 0.9% saline (2 ml/kg.), and these groups

served as controls. The four groups were killed, by decapitation, four hours after injection so that one group of α-MPT treated and one group of control rats were killed at 01.00 h. and the remaining groups at 13.00 h. The pineal glands were discarded and the brains were immediately removed, frozen in liquid nitrogen and stored at -14°C. 5-hydroxytryptamine was assayed by the method described in Chapter Three.

In a second experiment two groups of ten rats were injected intraperitoneally with α-MPT methyl ester (250 mg/kg. 2m/kg) eighteen hours and again four hours before decapitation. Two control groups of ten rats were injected intraperitoneally with 0.9% saline (2ml/kg), at the same times. The brains were removed at 01.00 h. and 13.00 h. as described previously and the 5-hydroxytryptamine content of the brain was assayed.

10.2.3 The Effects of 6-hydroxydopamine on the twenty-four hour variation of 5-hydroxytryptamine concentrations in the rat brain.

Male Sprague Dawley rats (60-80g) were maintained for ten days under the constant environmental conditions described in Chapter Two, so that the body weight of the rats was 130 - 150g. at the time of the start of experiment. In two groups of eight rats 6-hydroxydopamine (250 µg/rat in a volume of 10 µl), was injected into the lateral ventricle of the brain. Two groups of eight rats were similarly injected with 0.9% saline. The operations were carried out under ether anaesthesia. The rats were returned to the environmental cabinets for a further eighteen days, when they were killed by decapitation so that one group of 6-hydroxytryptamine treated and one group of control rats were killed at 01.00 h. and the

remaining groups at 13.00 h. The brains were removed and 5-hydroxytryptamine was assayed by the methods described previously.

10.3 RESULTS AND DISCUSSION

FLA-63 (25 mg/kg.) injected intraperitoneally was lethal to three rats from the groups killed at 01.00 h. and to two rats from the group killed at 13.00 h. No rats died as a result of the intraperitoneal injection of N/1 HCl in control groups. Rats in the experimental groups therefore died as a result of FLA-63. The results from the remaining rats are shown in Figure 56. There was no significant difference in brain 5-hydroxytryptamine concentrations between control and FLA-63-treated rats at 01.00 h. However, the concentration of the amine in FLA-63-treated rats was decreased by 42% when compared with control values at 13.00 h.

FLA-63 (25 mg/kg.) injected orally did not significantly alter 5-hydroxytryptamine concentrations at either 01.00 h. or 13.00 h. (Figure 57). However it cannot be concluded that FLA-63 has no effect in altering 5-hydroxytryptamine concentrations, since levels were decreased at 13.00 h. following intraperitoneal injection of the drug. Neither can it be concluded that this latter effect is due to the direct effects of the drug, since these effects were measured in animals whose cage mates had died as a result of FLA-63. Other factors such as stress and fear must be taken into account. These experiments were not repeated since lower doses of the drug have little effect on catecholamine concentrations (Anden and Fuxe 1971) and higher doses could only increase lethality. Since FLA-63 was unsatisfactory, and no better inhibitor of dopamine-β-hydroxylase is available, the experiments reported in this Chapter were unable to differentiate between the effects of noradrenaline and dopamine on the twenty-four hour rhythm of 5-hydroxytryptamine concentrations in the rat brain.

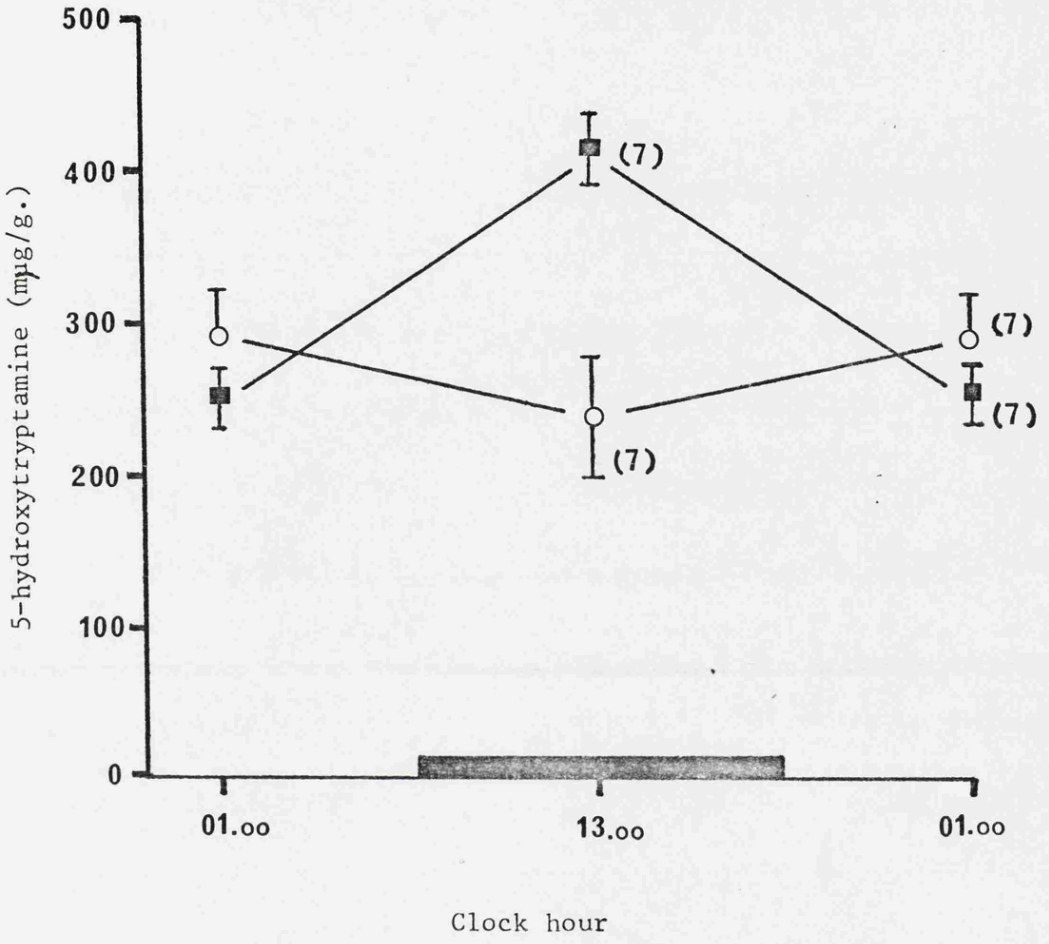


Figure 56. The effects of FLA-63 (25 mg/kg.i.p. O —— O) on the concentrations of 5-hydroxytryptamine (± SEM) in the rat brain measured at 01.00 h. and 13.00 h. (O ——O) (control ■ ——■).

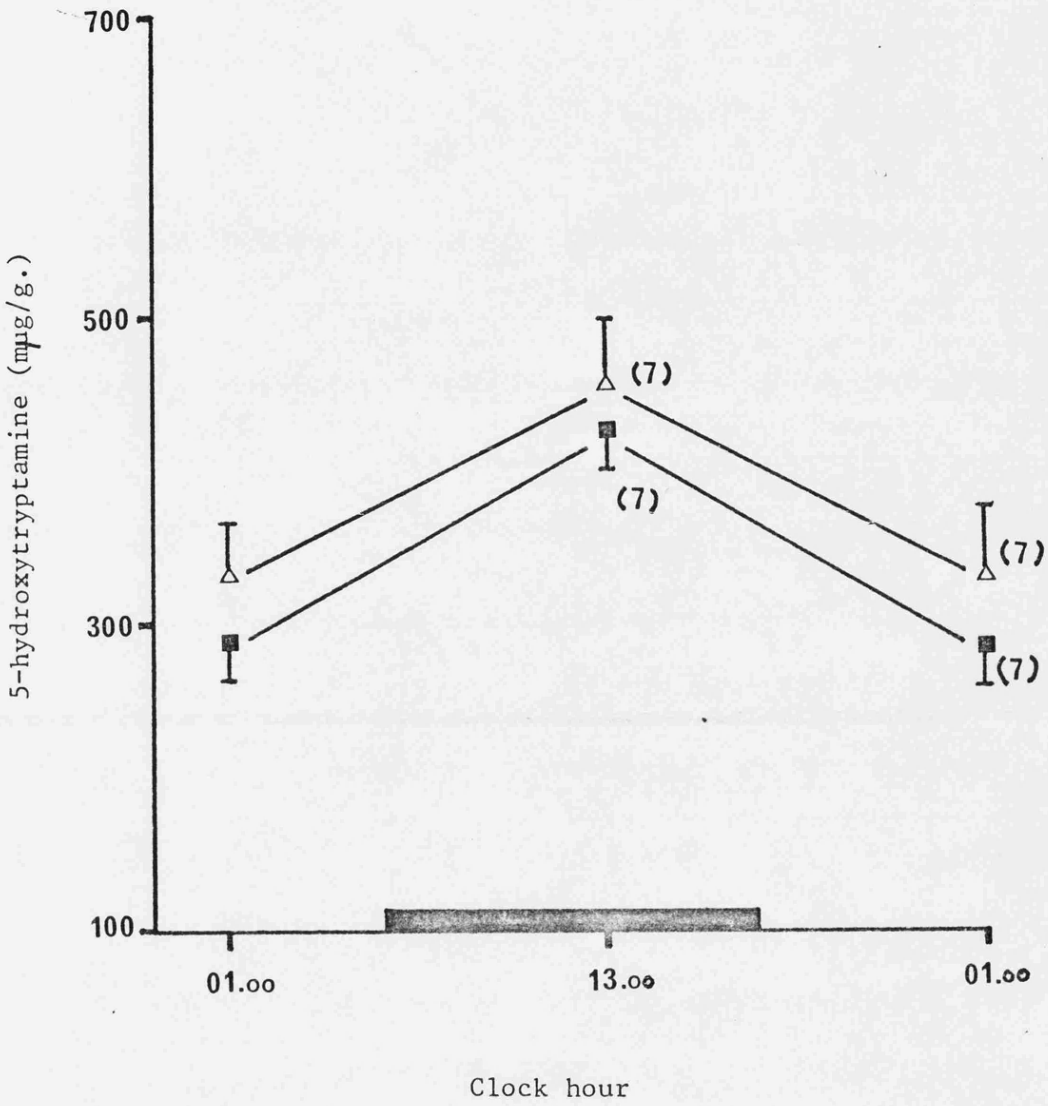


Figure 57. The effects of FLA-63 (25 mg/kg p.o. △——△) on the concentrations of 5-hydroxytryptamine (± SEM) in the rat brain measured at 01.00 h. and 13.00 h. (△——△) Control (■——■).

α-methyl-p-tyrosine (250 mg/kg. I/P) did not significantly alter 5-hydroxytryptamine concentrations at 13.00 h. when compared with saline injected controls (Figure 58). However, 5-hydroxytryptamine concentrations were significantly greater ($p < 0.01$) at 01.00 h. in -MPT-treated rats when compared with controls. It would therefore appear that the rate of decline of 5-hydroxytryptamine concentrations during the initial part of the dark period has been decreased following the inhibition of catecholamine synthesis with α-MPT.

In order to determine whether a similar response could be obtained at 13.00 h., two doses of α-MPT (250 mg/kg.I/P) were injected, the first 18 h. and the second 4 h. before the rats were killed (Figure 59). 5-hydroxytryptamine concentrations were significantly greater ($p < 0.001$) at both 01.00 h. and 13.00 h. The increase was 128% at 01.00 h. and 56% at 13.00 h. It would therefore appear that when the concentrations of catecholamines (dopamine and noradrenaline) are depleted in the rat brain by α-MPT the twenty-four hour rhythm of 5-hydroxytryptamine concentrations is abolished since concentrations at 01.00 h. and 13.00 h. were not significantly different.

The effects of the intraventricular injection of 6-OHDA on 5-hydroxytryptamine concentrations in the rat brain can be seen in Figure 60. Concentrations of the amine were significantly greater (72%) ($p < 0.001$) at 01.00 h., and (20%) ($p < 0.01$) at 13.00 h. eighteen days after 6-OHDA injection when compared with control rats.

The concentrations of 5-hydroxytryptamine were not significantly different at these two clock hours following 6-OHDA injections. It would therefore seem likely that central catecholamine-containing neurones play a part in the control of 5-hydroxytryptamine concentrations in the rat brain, since the effects of 6-OHDA are not likely to be toxic

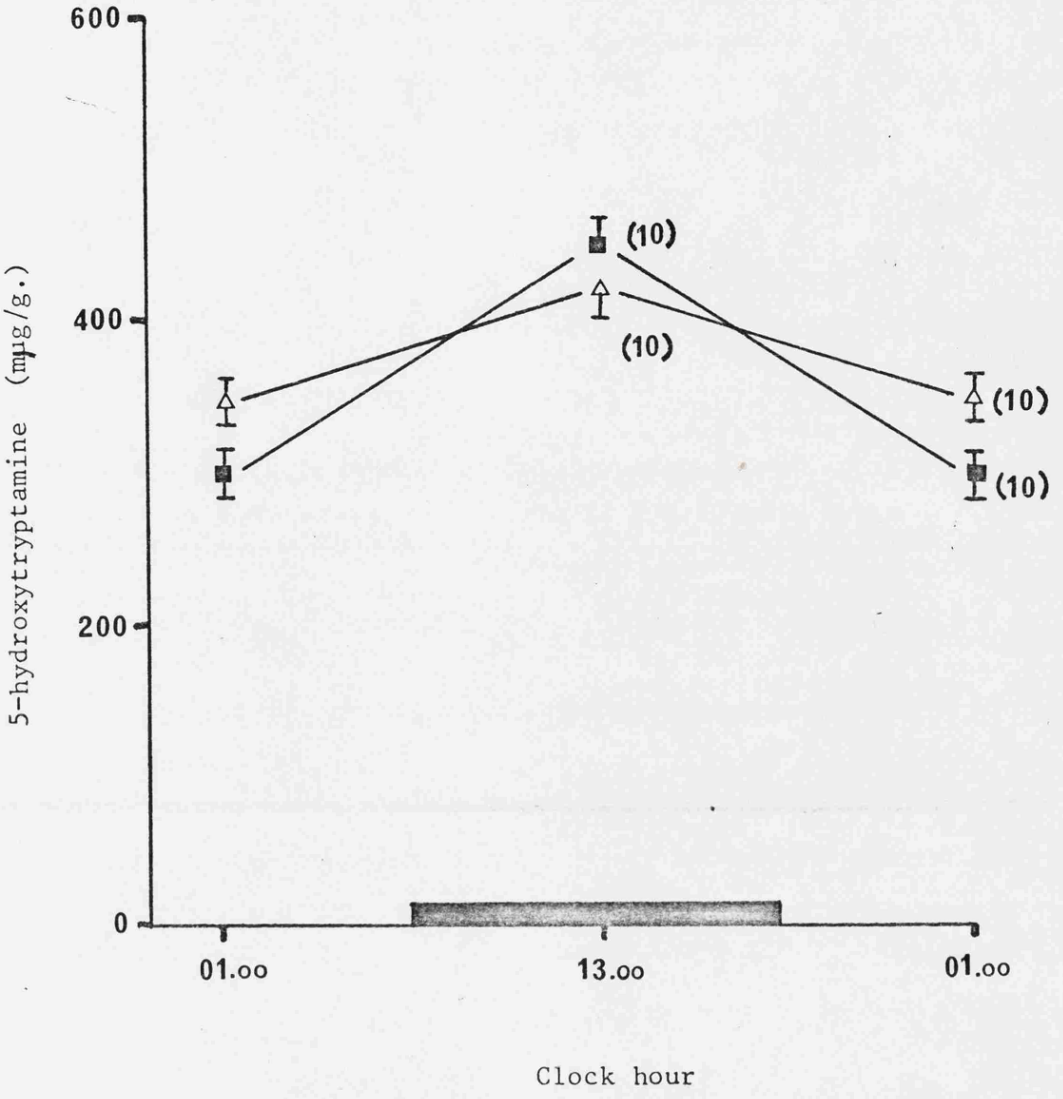


Figure 58. The effects of ∝-MPT (250 mg/kg i.p. Δ——Δ) administered 4 h. before the rats were killed, on the concentrations of 5-hydroxytryptamine (± SEM) in the rat brain measured at 01.00 h. and 13.00 h. (Δ——Δ). Control (■——■).

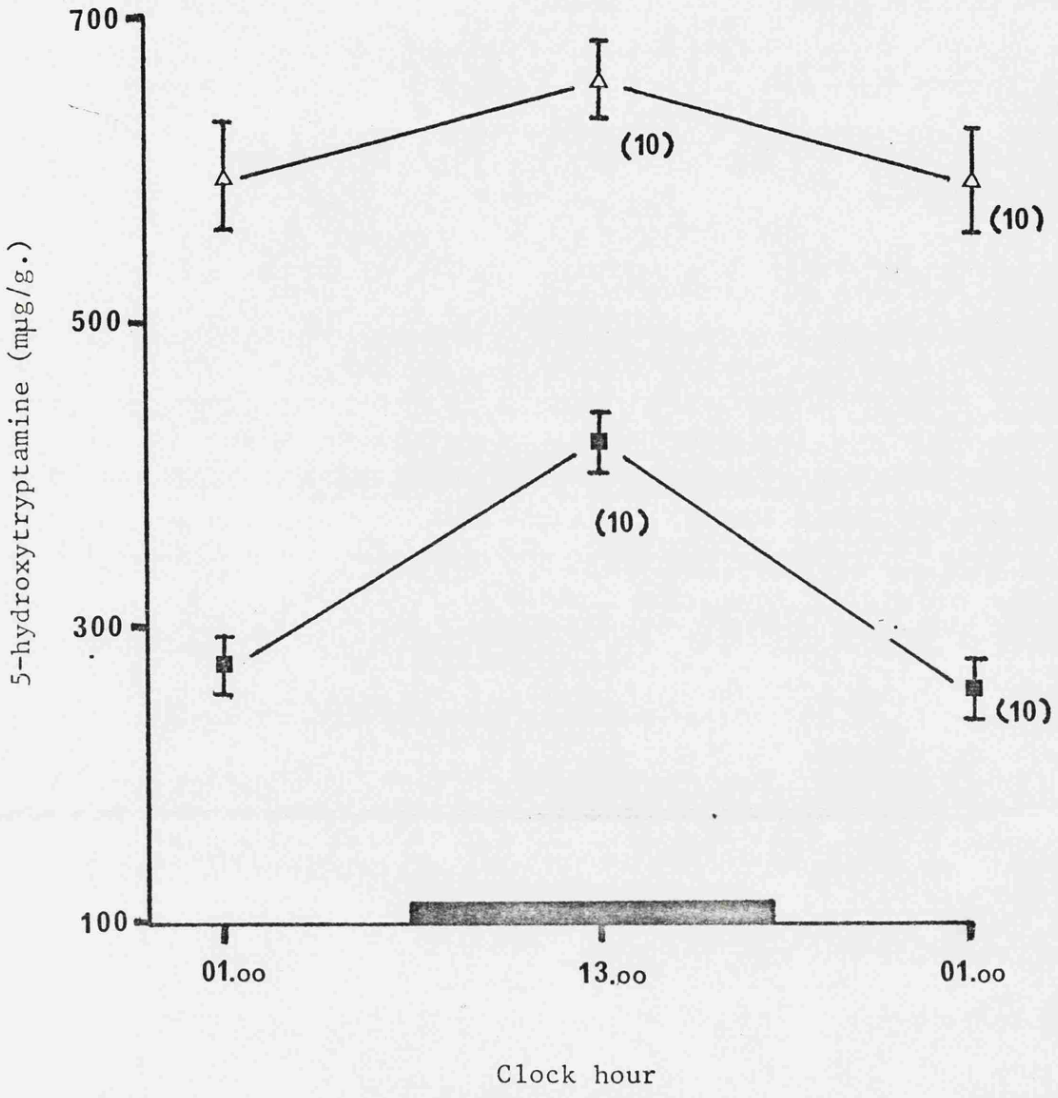


Figure 59. The effects of α-MPT (250 mg/kg.i.p.Δ——Δ) administered 18 h. and 4 h. before the rats were killed, on the concentrations of 5-hydroxytryptamine (± SEM) in the rat brain measured at 01.00 h. and 13.00 h. (Δ——Δ) Control (■——■).

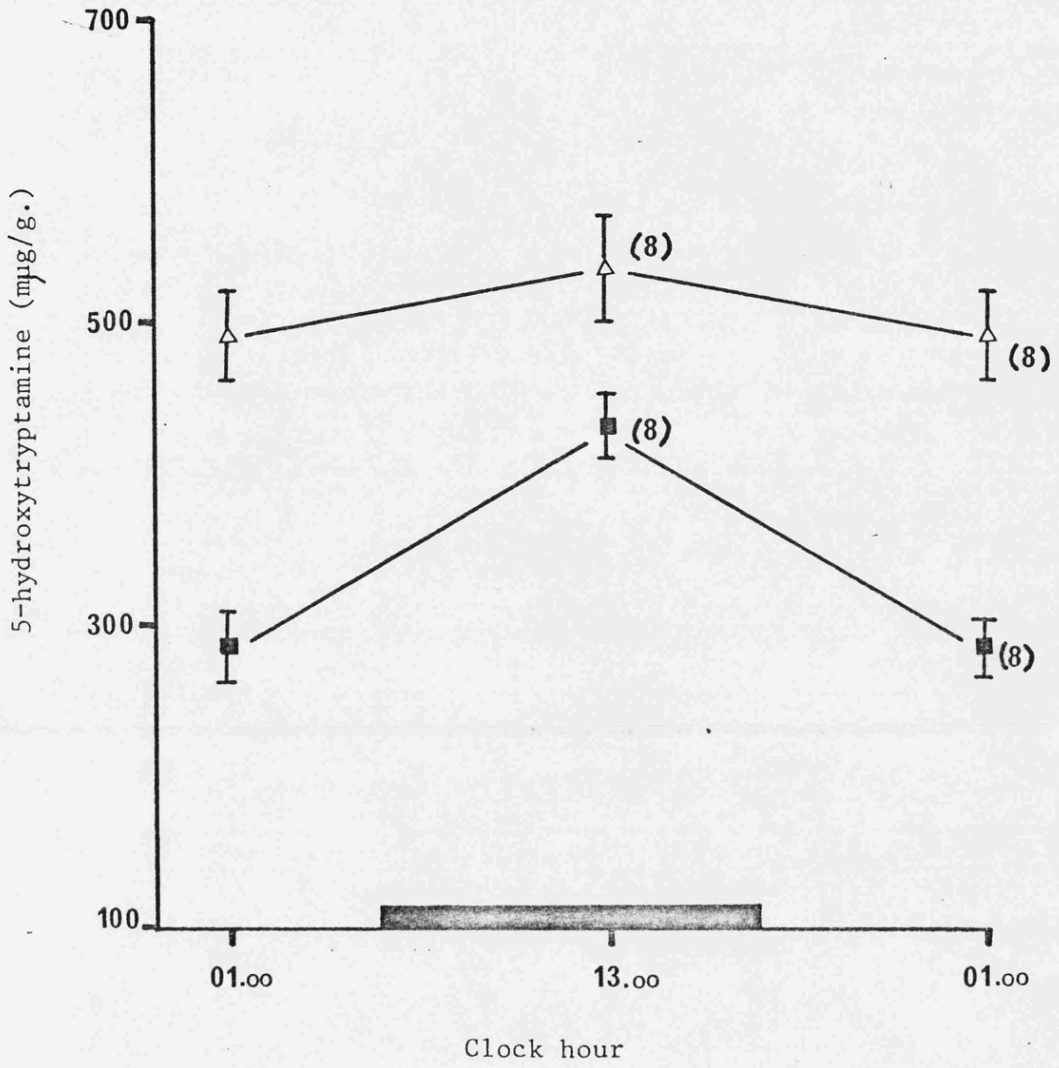


Figure 60. The effects of 6-OHDA (250 μg i.c.v. Δ——Δ) on the concentration of 5-hydroxytryptamine (± SEM) in the rat brain measured at 01.00 h. and 13.00 h. (Δ——Δ) (Control ■——■).

to 5-hydroxytryptamine containing neurones (see Introduction). However the alterations in 5-hydroxytryptamine concentrations noted here could be caused either by an increased sensitivity of noradrenaline receptors or by behavioural changes in the rats.

If the effects of α-MPT and 6-OHDA are compared, it is apparent that the catecholamines may play an important part in the control of the synthesis of 5-hydroxytryptamine, and since these effects are most marked at 01.00 h. (Figures 59,60) it is possible that lowered concentrations of the catecholamines could represent a decreased inhibition of 5-hydroxytryptamine synthesis. Further implications of this supposition will be discussed in Chapter Twelve.

CHAPTER ELEVEN

Locomotor activity and 5-hydroxytryptamine concentrations in the rat brain.

11.1 INTRODUCTION

Locomotor activity has been speculatively correlated with 5-hydroxytryptamine concentrations in the rat brain (Grahame-Smith 1971), and the twenty-four hour rhythm in locomotor activity has been widely reported (Richter 1922; Hunt and Schlosberg 1939; Peacock, Hodge and Thomas 1966; Hutchins and Rogers 1970). The rat is a nocturnal animal which exhibits a high degree of spontaneous activity commencing shortly after the onset of darkness, and almost all locomotor activity occurs during the dark period. Locomotor activity depends on a large number of factors including age of the animal, hunger, thirst and the presence of external stimuli such as noise and temperature. Richter (1922) reported six to ten bursts of activity during the dark period, however there is a large difference in the amount of activity, even between littermates (Reed 1947).

In this Chapter the effects of various drugs known to alter concentrations of 5-hydroxytryptamine in the rat brain, have been compared, by their effects on the locomotor activity of the rat.

11.2 METHODS AND MATERIALS

Male Sprague Dawley rats (80 - 100g) were maintained under constant environmental conditions in a light-proofed and sound-proofed room lit by a 150 watt electric lamp connected to a time switch such that the light came on at 06.00 h. and switched off at 18.00 h. The room was thermostatically controlled and maintained at 23 $\pm$ 3°C.

Each rat was housed individually in a "Squirrel cage" (E.K. Bowman Ltd., London) with access to food and water ad libitum. Each "Squirrel cage" consisted of an activity wheel (16 in. x 5 in.) connected to a nesting box (8 in. x 10 in.) by an open portal. Each "Squirrel cage" was fitted with a device for recording the drinking activity of the animal (for full details see Navaratnam 1974). Locomotor activity was measured by the closing of an electrical contact with each revolution of the activity wheel, and electrical pulses were counted and stored in an Elmeg DMO 12 multi-channel digital printing aggregate unit and visualised on the Elmeg 2DG V 12 channel digital print-out unit (both instruments were manufactured by Elektro-Mechanik GmbH, Germany). Under this procedure each rat was not isolated from external stimuli i.e. each animal, although not within sight of its neighbours, was subjected to the auditory stimuli produced by the revolutions of neighbouring activity wheels. However, by randomising the drug treatment given to animals in each cage, it was hoped that any such extraneous effects would be nullified. The drinking monitor and the recording devices for measuring locomotor activity were assembled outside the experimental room in order to keep auditory stimuli to a minimum.

Groups of nine rats were housed in the experimental room and were left undisturbed until each exhibited a synchronised locomotor activity rhythm, (usually after 12-15 days). Drugs were then administered either in the drinking water or intraperitoneally, and the effect of the drugs on locomotor activity was monitored until control levels were reached or until six days following drug administration whichever was the sooner.

The effects of the following drugs were determined:- tryptophan, 5-hydroxytryptophan, p-chlorophenylalanine, isocarboxazid, α-methyl-p-tyrosine, and tap water or saline as a control. The effects of these drugs on locomotor activity were compared with their effects on the concentration of 5-hydroxytryptamine in the rat brain described in previous chapters in this thesis.

11.2.1 Expression of Results

In only two treatments (isocarboxazid and p-chlorophenylalanine) were the results sufficiently uniform to calculate a mean result from the data obtained. The results from these two treatments are therefore expressed as the mean effect of drug treatment on locomotor activity per clock hour, and the graphs shown (Figures 62 and 63) are drawn from the day of maximum effect of the drugs (day 3 for p-chlorophenylalanine and day 5 for isocarboxazid). Locomotor activity has been scaled as follows:

Scale 1.	0	-	49 r.p.m.
2.	50	-	199 r.p.m.
3.	200	-	499 r.p.m.
4.	500	-	999 r.p.m.
5.	1000	-	1499 r.p.m.
6.	1500	-	2000 r.p.m.

In other experiments results were too variable to express on this basis. The gross locomotor activity was therefore expressed per twenty-four hours and plotted for each animal three days before and six days after the start of drug treatment. Each animal therefore acted as its own control and results are expressed as the percentage change in locomotor activity with the activity on the first day of treatment representing 100%.

11.3 RESULTS AND DISCUSSION

In the first experiment a monoamine oxidase inhibitor, isocarboxazid, was given to rats in the drinking water at a concentration of 100 µg/ml. Control rats received normal tap water. The total locomotor activity per hour was plotted against clock hour (Figure 61). Rats which drank tap water demonstrated a biphasic locomotor activity rhythm, with minimal activity during the light period and a subsequent increase at the onset of darkness. This level was maintained throughout the dark period. In rats drinking the isocarboxazid solution a similar rhythm was displayed but the level of activity during the dark period was increased by more than 100%. No increase in locomotor activity was recorded during the light period.

When these results were compared with the effects of isocarboxazid on brain 5-hydroxytryptamine concentrations (Chapter Eight, Figures 51,52) it was apparent that the increased concentrations of 5-hydroxytryptamine during the light period could be inversely correlated with the decreased locomotor activity during this period, while decreased concentrations of the amine may be inversely correlated with the marked increase in activity during the dark period.

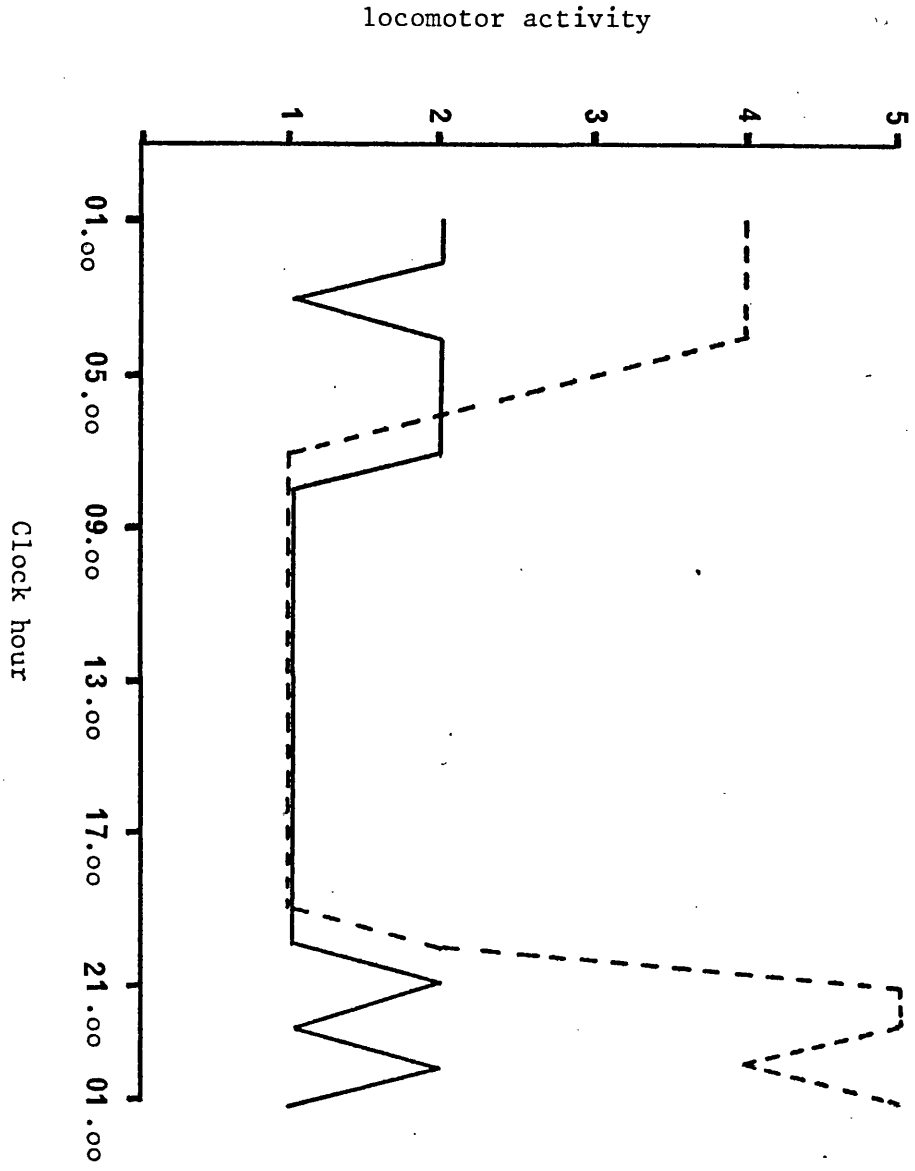


Figure 61. The effects of isocarboxazid (100 µg/ml. administered in the drinking water) on the locomotor activity of the rat (----isocarboxazid; ——— control).

In the second experiment rats were injected orally with either p-chlorophenylalanine (316 mg/kg) or saline (0.9%). The saline injected rats demonstrated a similar pattern of locomotor activity to the control rats in the previous experiment. In p-CPA injected rats the rhythm of locomotor activity was completely disrupted (Figure 62), with activity throughout the twenty-four hours at similar levels to control rats during the dark period. Maximum disruption was demonstrated three and four days after the injection of p-CPA and this disruption may therefore be related to the decrease of 5-hydroxytryptamine concentrations found at a similar time (Chapter Five). A further inverse correlation between locomotor activity and 5-hydroxytryptamine concentrations has therefore been shown.

Figure 63 demonstrates the drinking activity of a group of nine rats housed in squirrel cages, and is typical of the activity recorded throughout these experiments. In the first three columns rats had been injected with saline, in the second three with p-CPA and the final three received isocarboxazid in the drinking water. It was apparent that virtually all the drinking activity occurred during the dark period and very little occurred during the light period even following disruption of the locomotor activity rhythm following p-CPA injection.

The effects of the two precursors of 5-hydroxytryptamine synthesis (tryptophan and 5-hydroxytryptophan) on the locomotor activity rhythm of the rat are shown in Figures 64 and 65. Each point represents the gross activity of one animal per twenty-four hours. The effects were somewhat variable from animal to animal, but the overall picture is similar for both compounds in that a

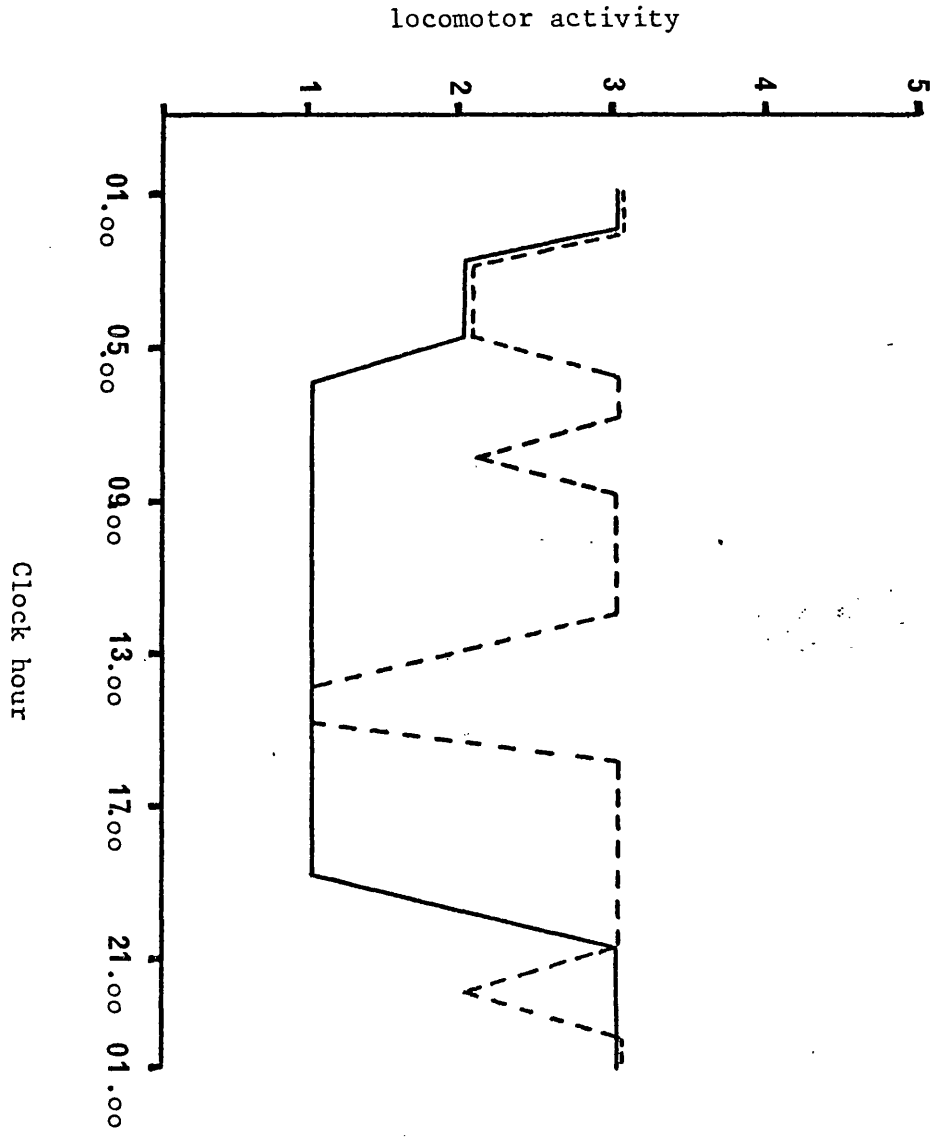
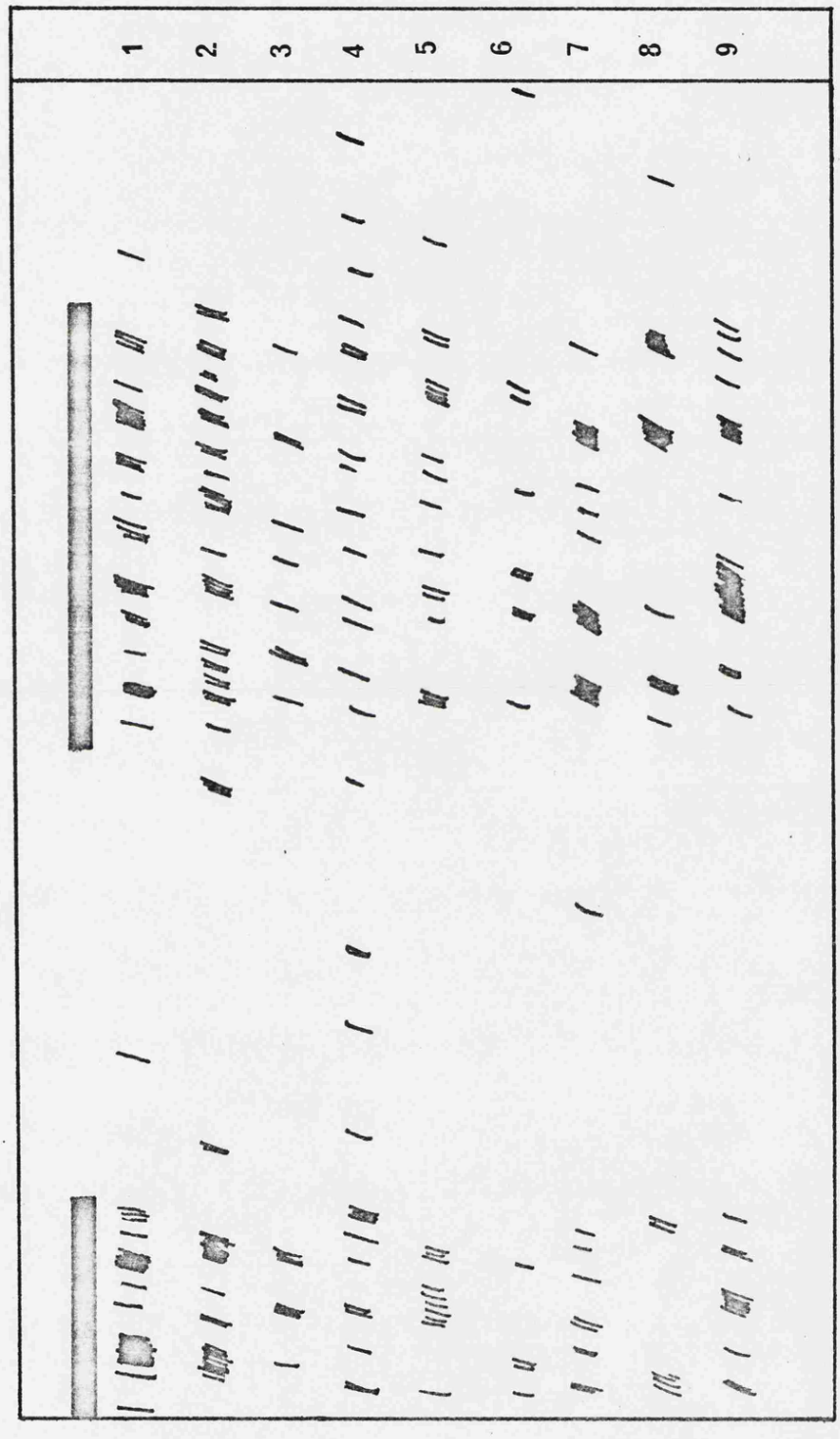


Figure 62. The effects of p-CPA (316 mg/kg.p.o) on the locomotor activity of the rat (---- p-CPA, —— Control).



1 - 3 Tap water
4 - 6 p-CPA p.o.
7 - 9 Isoboxazid drinking water

Figure 63. A record of the drinking activity of rats over a 36 h. period. Each stroke represents one period of drinking.

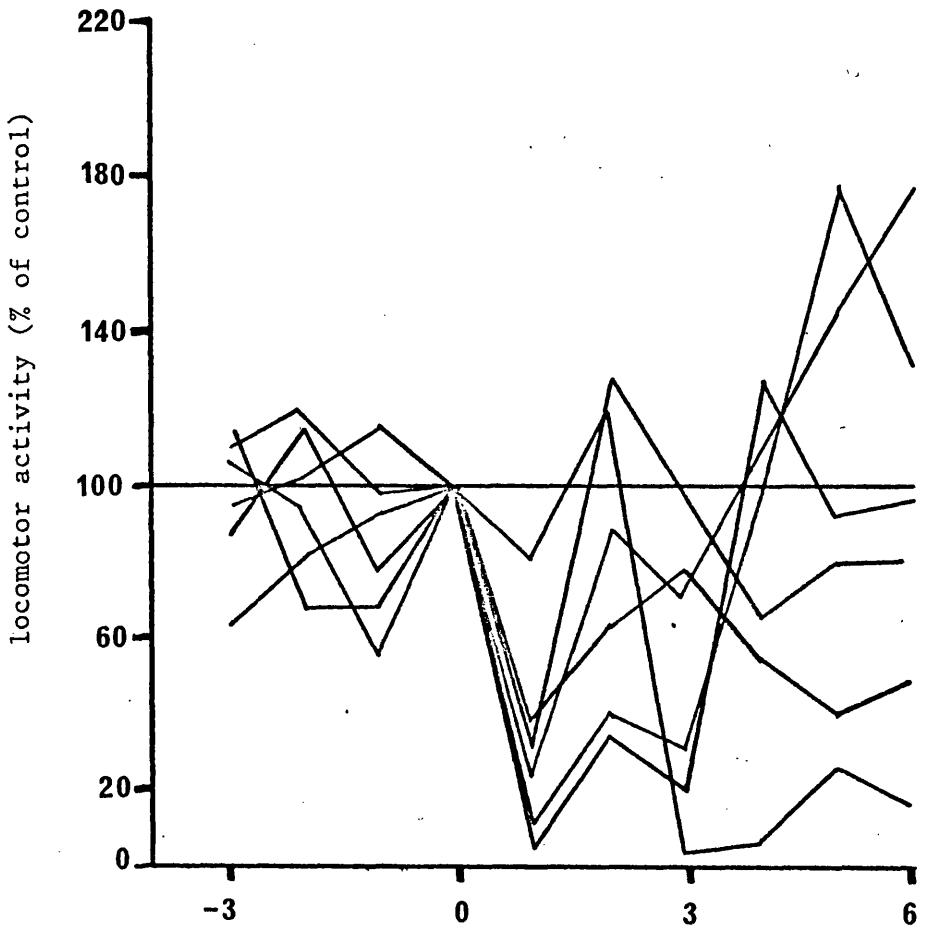


Figure 64. The effects of tryptophan (1 mg/ml. administered in the drinking water) on the locomotor activity of the rat.

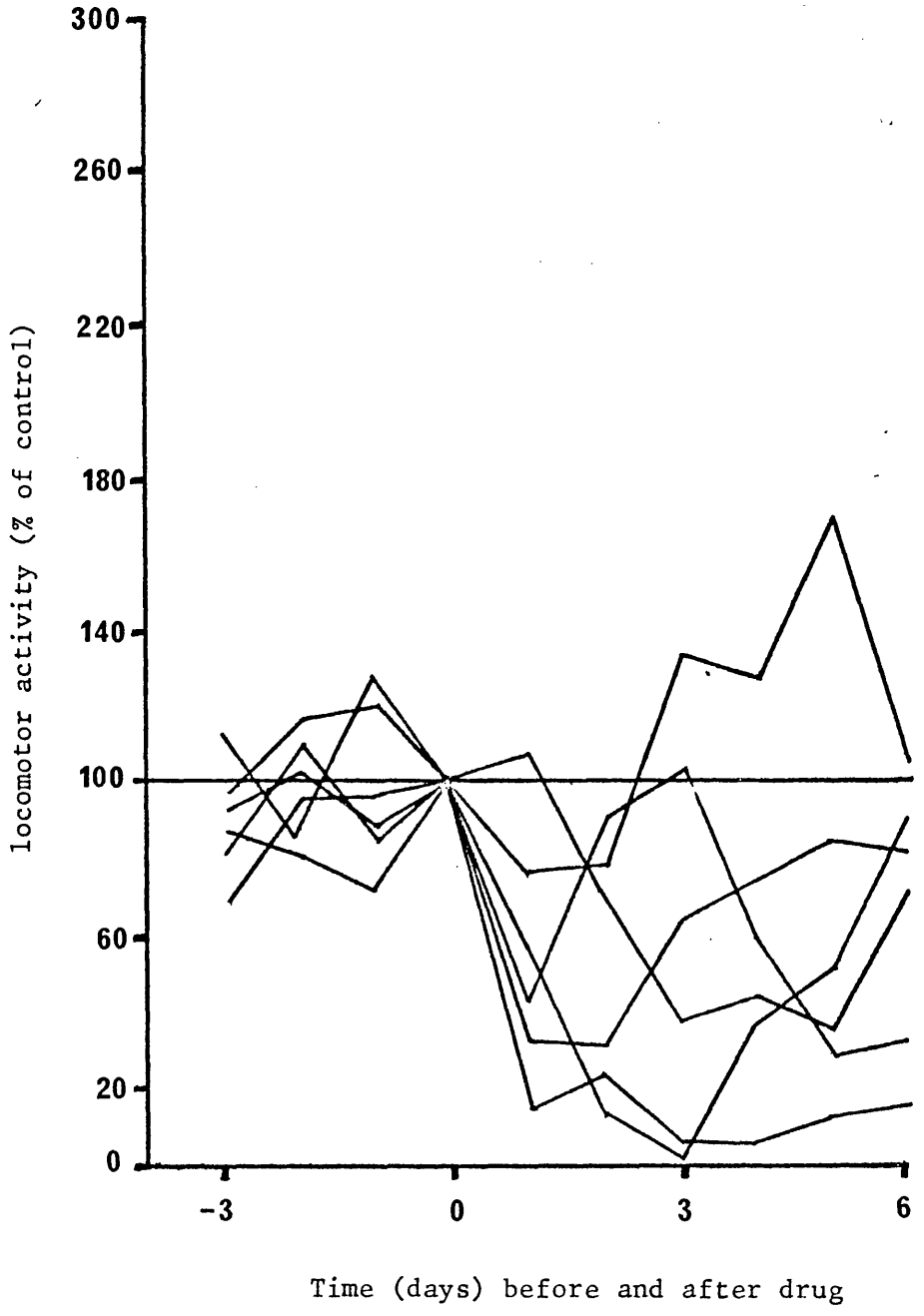


Figure 65. The effects of 5-hydroxytryptophan (1mg/ml administered in the drinking water) on the locomotor activity of the rat.

general depression of locomotor activity was seen throughout the experiment. Both drugs were given in the drinking water at a concentration of 1 mg/ml. There were six animals in each group.

From evidence available, it is almost certain that tryptophan and 5-hydroxytryptophan are rapidly metabolised in the body, and only a small amount of that ingested will be taken up by the CNS. It must also be borne in mind that the rats only consumed these compounds during the dark period. When these facts are considered together it seems likely that a high degree of variability in the effects on locomotor activity is bound to exist between animals, since the amount of tryptophan or 5-hydroxytryptophan ingested at any given time during the dark period is unlikely to be the same in any of the animals. Drugs which have longer lasting effects (p-CPA, isocarboxazid) are more likely to produce consistent results between animals.

From these experiments it can be seen that drugs which elevate only brain 5-hydroxytryptamine concentrations (tryptophan and 5-hydroxy-tryptophan) also decrease locomotor activity, while drugs which elevate all amine concentrations (isocarboxazid) increase activity. It was therefore interesting to measure the effects of a drug which depletes catecholamine concentrations but increases 5-hydroxytryptamine concentrations i.e. α-MPT (Chapter Ten, Figure 59). α-MPT was injected intraperitoneally (250 mg/kg) on two consecutive days at 13.00 h. and the results recorded (Figure 66). The results were again very variable but it appeared that locomotor activity was initially reduced and subsequently increased although this latter effect may have been due to the effects of the drug wearing off (a rebound effect). It is proposed that the initial reduction of locomotor activity is a result of the increased 5-hydroxytryptamine concentrations induced by α-MPT.

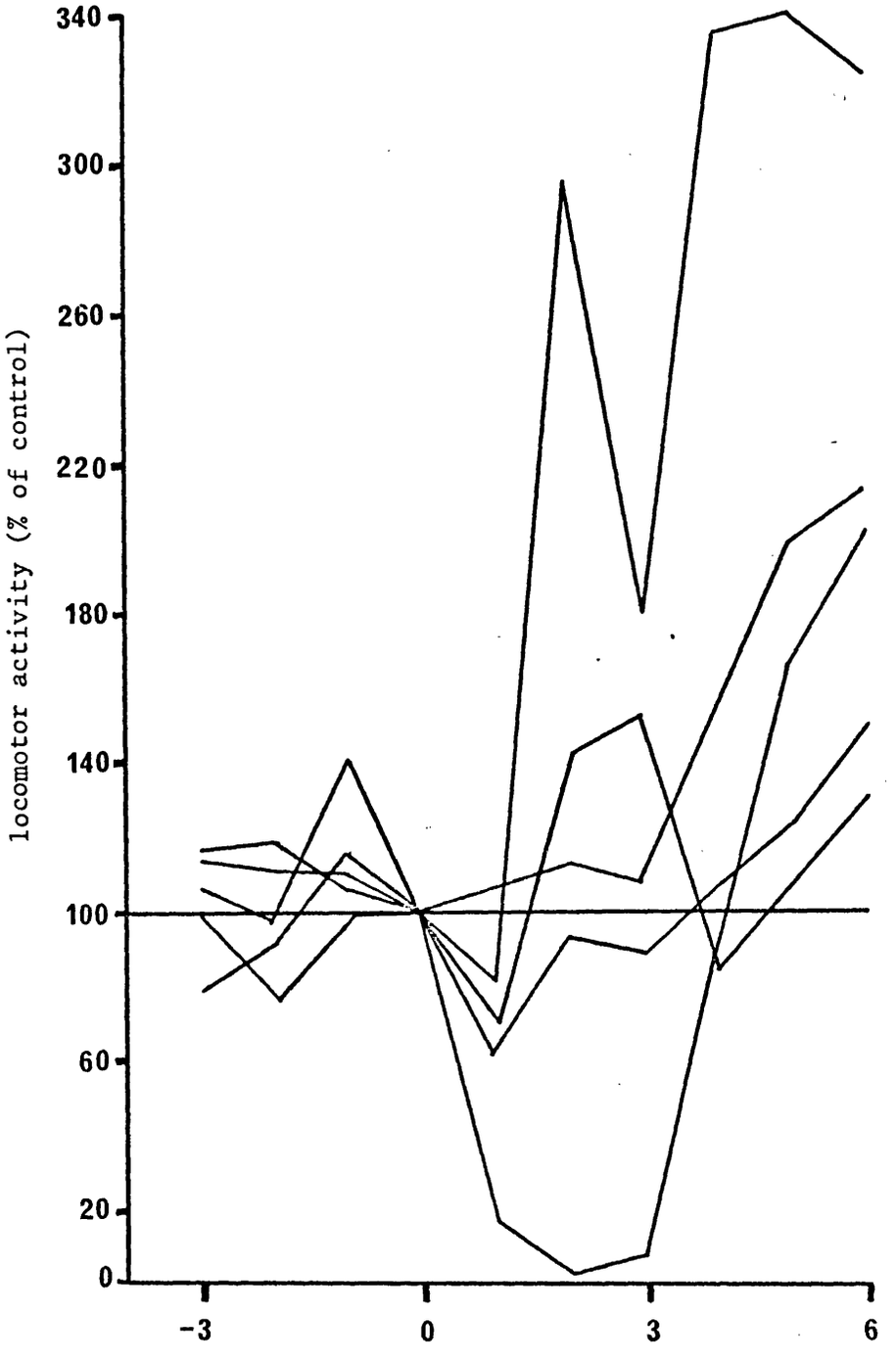


Figure 66. The effects of α-MPT (250 mg/kg i.p. injected on two consecutive days at 13.00 h.) on the locomotor activity of the rat.

It seems likely from these experiments that 5-hydroxytryptamine concentrations play a part in controlling locomotor activity, with the major action being a decrease in activity as 5-hydroxytryptamine concentrations increase.

C H A P T E R T W E L V E

Discussion

12. DISCUSSION

Discussions directly related to the results obtained during the course of this work, and the relationship between these results and those of other workers have been presented in the relevant Chapters in this thesis and will not be reiterated here. However the conclusions which can be drawn from these results will be collected together to present an argument which will suggest the identity of the regulating factors in the twenty-four hour rhythm of 5-hydroxytryptamine concentrations in the rat brain. This hypothesis will hinge on the fact that a twenty-four hour rhythm of 5-hydroxytryptophan decarboxylase activity has been detected and that an interaction between catecholamines and 5-hydroxytryptamine has been demonstrated.

From the results reported in this thesis and from those reported by other workers (Chapter Three) there would appear to be no doubt that the concentrations of 5-hydroxytryptamine found in the rat brain vary with the time of day. This variation is normally entrained to produce a twenty-four hour rhythm, with highest concentrations during the light period (13.00 h.) when the rats are least active, and lowest concentrations during the dark period (01.00 h.) when the rats are most active. Highest concentrations exceeded lowest concentrations by 40% (Chapter Three).

Having established this, it is necessary to decide which of the many possible mechanisms do actually play a part in controlling the rhythm. The possible means of attaining maximum concentrations of 5-hydroxytryptamine in the rat brain at 13.00 h. can be summarised as follows:

1) A reduced release of 5-hydroxytryptamine from 01.00 h. to 13.00 h. subsequently increasing after this period.

2) A reduced rate of intracellular metabolism as concentrations of the amine increase, and an increased rate as the concentrations of 5-hydroxytryptamine decrease.

3) An enhanced synthesis of the amine from 01.00 h. to 13.00 h. subsequently decreasing after this period.

Of these possibilities the first has already been questioned (Chapter Nine) since the concentrations of 5-hydroxyindole-3-acetic acid in the rat brain vary with clock hour such that highest concentrations were recorded at the same clock hour as the highest concentrations of 5-hydroxytryptamine. In addition, the activity of monoamine oxidase does not vary throughout the twenty-four hours (Chapter Eight). It therefore follows that 5-hydroxyindole-3-acetic acid is formed as a result of the 5-hydroxytryptamine released from nerve terminals. The increase of 5-hydroxytryptamine concentrations from 01.00 h. to 13.00 h. cannot therefore be controlled by a reduced release of 5-hydroxytryptamine during this period.

The fact that monoamine oxidase activity does not vary with clock hour (Chapter Eight) also rules out the second possibility. The suggestion of feedback control of the synthesis of 5-hydroxytryptamine can also be ruled out at the physiological concentrations involved, since concentrations of 5-hydroxytryptamine were maintained at elevated levels in the presence of monoamine oxidase inhibitors (Chapter Eight, Figure 51).

If one considers the third possibility i.e. variations in the synthesis of 5-hydroxytryptamine with clock hour, then it is apparent that a large number of factors could be involved. Evidence for this possibility may be summarised as follows:

1) 5-hydroxytryptamine concentrations did not increase during the light period following the inhibition of tryptophan-5-hydroxylase with p-CPA (Chapter Five).
2) The uptake of tryptophan into a synaptosomal preparation was greater at 13.00 h. than at 01.00 h. (Chapter Four).
3) The activity of 5-hydroxytryptophan decarboxylase was higher at 13.00 h. and 17.00 h. than during the dark period (Chapter Seven).
4) The synthesis of 5-hydroxytryptamine was greater at 13.00 h. than at 01.00 h. following the inhibition of monoamine oxidase with either pargyline or tranylcypromine (Chapter Eight).

Other factors in the synthesis of 5-hydroxytryptamine are not integral to the main argument, but must also be considered, and are listed as follows:

1) Tryptophan concentrations in the brain parallel the concentrations of the amino acid in the serum, whether or not this is bound to albumin (Chapter Four, Figure 24). No evidence has been produced in this thesis to determine which tryptophan fraction, if either, controls the concentrations of tryptophan in the brain. However, the discovery of a 180 degree phase difference between the twenty-four hour rhythms of brain tryptophan concentrations and brain 5-hydroxytryptamine concentrations strongly suggests that endogenous tryptophan concentrations do not control the twenty-four hour rhythm of 5-hydroxytryptamine concentrations in the rat brain. Indeed it is further suggested that the concentrations of 5-hydroxytryptamine in the brain must be

buffered from the possible effects of fluctuating tryptophan concentrations.

This latter assumption may not be viable since it is not known how much endogenous tryptophan in the brain is available for uptake into neurones or nerve cell bodies. In fact a knowledge of the localisation of the tryptophan available for uptake would greatly clarify the situation. However, on the present evidence produced, endogenous concentrations of tryptophan in the brain are not considered to play a part in the production of the twenty-four hour rhythm of 5-hydroxytryptamine concentrations in the rat brain.

2) The uptake of ^{14}C-tryptophan into brain region homogenates did not vary with clock hour (Chapter Four, Figures 30,31), although a higher rate of uptake was recorded at 13.00 h. in an isolated synaptosomal preparation (Figure 29). Thus a higher initial accumulations of amino acid precursor occurred at the same clock hour as highest concentrations of 5-hydroxytryptamine in the brain. Not only is this important in that higher initial accumulation of precursor could lead to a higher rate of synthesis, but might also provide the mechanism by which the effects of the variation in endogenous tryptophan concentrations are nullified.

3) Tryptophan-5-hydroxylase activity does not vary with clock hour (Chapter Five), and it is therefore unlikely that the activity of this enzyme participates in the production of the twenty-four hour rhythm in 5-hydroxytryptamine concentrations. However it is recalled that tryptophan-5-hydroxylase exists in both a soluble and particulate form

(Knapp and Mandell 1972) with the former predominating in cell bodies and axons and the latter in nerve terminals. Since a twenty-four hour variation of tryptophan uptake was indicated in an isolated nerve ending preparation but not in a crude homogenate it is not inconceivable that the particulate form of the enzyme may produce more 5-hydroxytryptophan at 13.00 h. than at 01.00 h. If such a variation does occur it was not detected by the methods employed in this thesis and has not been reported in the literature.

4) The uptake of 5-hydroxytryptophan into nerve ending homogenates does not vary with clock hour (Chapter Six, Figures 41,42), and cannot therefore be considered to have a role in the production of the twenty-four hour rhythm of 5-hydroxytryptamine. In addition it has been shown that the availability of 5-hydroxytryptophan for conversion to 5-hydroxytryptamine does not play a part in the production of the rhythm (Chapter Seven) since inhibition of tryptophan-5-hydroxylase, and therefore inhibition of the availability of 5-hydroxytryptophan from brain tryptophan, by p-CPA did not alter the rates at which 5-hydroxytryptophan decarboxylase was able to convert 5-hydroxytryptophan to 5-hydroxytryptamine in vitro (Figures 43,48).

It is clear that of all the parameters measured, only the variation of 5-hydroxytryptophan decarboxylase activity with clock hour (Chapter Seven) can convincingly fill the role of a regulating factor in the production of the twenty-four hour rhythm of 5-hydroxytryptamine concentrations.

The rate of conversion of 5-hydroxytryptophan to 5-hydroxytryptamine varied with clock hour such that the rate at 13.00 h. was significantly greater than that at 01.00 h. (Figure 43). This difference is sufficient to account for the variation in 5-hydroxytryptamine concentrations at these clock hours, and it is therefore suggested that this factor plays a major role in the production of the twenty-four hour rhythm of 5-hydroxytryptamine concentrations in the rat brain.

The variation in the ability of 5-hydroxytryptophan decarboxylase to convert 5-hydroxytryptophan to 5-hydroxytryptamine is not due to rhythmic activity of the enzyme itself since the activity of the enzyme was similar at 01.00 h. and 13.00 h. when measured in a purified enzyme extract (Chapter Seven, Figure 47), nor is the variation due to the availability of pyridoxal phosphate or to the enzyme being saturated with substrate (Figure 46). The number of factors remaining which could alter the rate of production of 5-hydroxytryptamine are small. Other co-factors e.g. metal ions are a possible variable, but it seems unlikely that such components could be maintained in concentrations which would have to be so delicately balanced to produce these effects. A second possibility is competition for the enzyme by different substrates since a large number of amino acids can be taken up by 5-hydroxytryptamine containing neurones. Of particular interest is the competition between dihydroxyphenylalanine (DOPA) and 5-hydroxytryptophan (Chapter One), since it has been shown that 5-hydroxytryptamine concentrations decline as noradrenaline concentrations increase in the brain (or vice versa) (Chapter Ten), and noradrenaline concentrations exhibit a marked circadian rhythm which is 180^{o} out of phase with the twenty-four hour rhythm of 5-hydroxytryptamine concentrations.

It has been reported that L-DOPA decreases the synthesis and increases the deamination of 5-hydroxytryptamine (Karobath, Diaz and Huttunen 1972), and that DOPA and 5-hydroxytryptophan may compete for the same enzymic site (Yuwiler, Geller and Eiduson 1960). These authors also found that DOPA combines irreversibly at 5-hydroxytryptophan sites while acting elsewhere but that 5-hydroxytryptophan cannot combine irreversibly at DOPA sites. It is therefore apparent that aromatic-L-amino acid decarboxylase shows some degree of preference for the conversion of DOPA over 5-hydroxytryptophan.

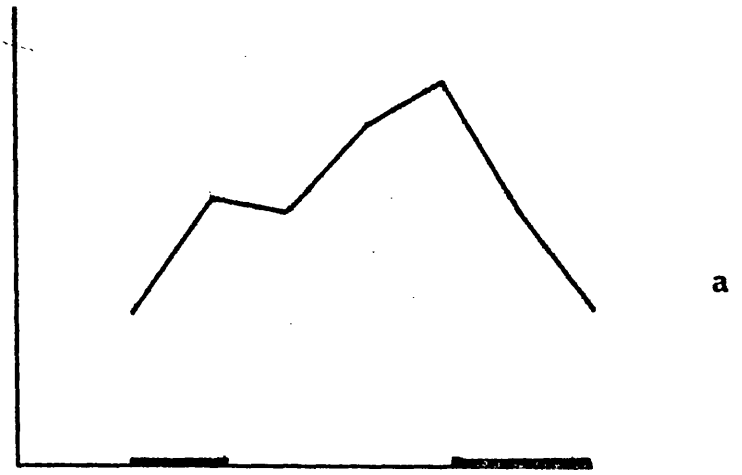
Since noradrenaline concentrations exhibit a twenty-four hour rhythm with highest concentrations during the dark period (Chapter Ten) and both tyrosine and phenylalanine concentrations exhibit circadian rhythms with highest concentrations at the same time (Fernstrom, Larin and Wurtman 1973), it seems likely that DOPA concentrations also exhibit a rhythm in this phase, particularly since noradrenaline concentrations are controlled by end-product inhibition of tyrosine hydroxylase (Lin, Neff, Ngai and Costa 1969). It therefore seems probable that the balance between the two competing substrates (DOPA and 5-hydroxytryptophan) will swing in favour of DOPA during the dark period.

As a consequence the conversion of 5-hydroxytryptophan to 5-hydroxytryptamine will decrease as the synthesis of noradrenaline increases.

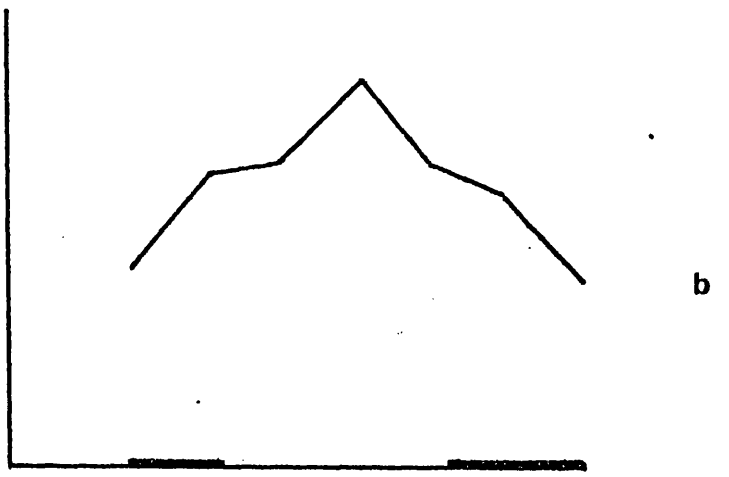
The problem then arises as to how DOPA concentrations are decreased so that 5-hydroxytryptamine synthesis increases. It is suggested that the rate of synthesis of noradrenaline is dependent on the activity of noradrenaline containing neurones ie. when these neurones are inactive the amine accumulates so that end product inhibition of tyrosine hydroxylase occurs and the synthesis of noradrenaline (and consequently the concentration of DOPA) decreases until such a time as the neurones

are reactivated. Some evidence has been obtained to support this hypothesis. In Chapter Eight it has been shown that 5-hydroxytryptamine concentrations continue to increase following monoamine oxidase inhibition, and it has been reported that 5-hydroxytryptamine concentrations increase to a much greater extent than noradrenaline concentrations following inhibition of this enzyme (Lin, Neff, Ngai and Costa 1969). In addition it has been reported that non-hydrazine monoamine oxidase inhibitors increase the rate of synthesis of 5-hydroxytryptophan to 5-hydroxytryptamine (Weber 1966). Further evidence has been presented in Chapter Ten, where it was shown that the injection of doses of α-MPT and 6-OHDA which reduce catecholamine concentrations to low levels (Spector, Sjoerdsma and Udenfriend 1965, and Hery, Rouer and Glowinski 1973 respectively) also increased the concentrations of 5-hydroxytryptamine in the brain. (Figures 59, 60), to abnormally high levels. In addition the twenty-four hour rhythm of 5-hydroxytryptamine in the brain was abolished by the injection of both monoamine oxidase inhibitors and inhibitors of catecholamine synthesis. These results are in agreement with the suggestion that the control of the twenty-four hour rhythm of 5-hydroxytryptamine concentrations is at least in part regulated by the ratio of the concentrations of DOPA to 5-hydroxytryptophan, and that the lower concentrations of 5-hydroxytryptamine recorded in rats during the dark period are a result of the decreased synthesis of the amine at this time.

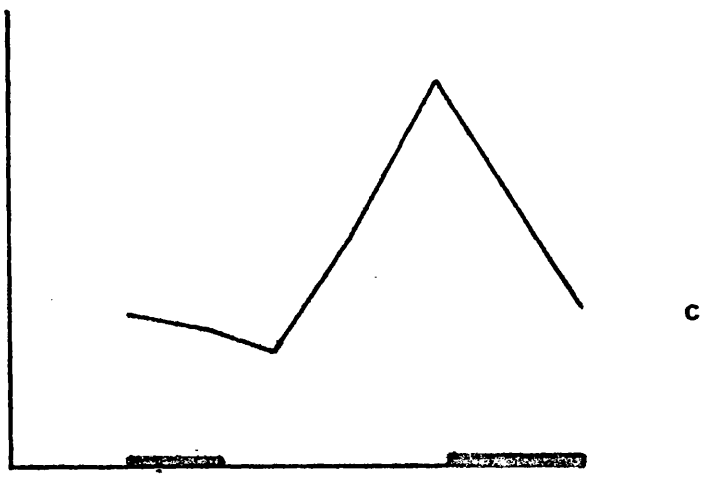
A comparison of the twenty-four hour rhythms of 5-hydroxytryptamine concentrations, 5-hydroxytryptophan decarboxylase activity and 5-hydroxyindole-3-acetic acid concentrations (Figure 67) shows that the results produced in this thesis support the proposed hypothesis, but such a comparison also indicates that 5-hydroxytryptophan decarboxylase activity is not the only factor infuencing 5-hydroxytryptamine concentrations.



5-hydroxyindole-3-acetic acid



5-hydroxytryptamine



5-hydroxytryptophan decarboxylase

Figure 67. A comparison of the shapes of the twenty-four hour rhythms of (a) 5-hydroxyindole-3-acetic acid (b) 5-hydroxytryptamine and (c) 5-hydroxytryptophan decarboxylase activity.

When these rhythms are compared (Figure 67) it can be seen that the rhythm of 5-hydroxyindole-3-acetic acid is directly dependent on the other two. Also the shoulder which is seen in the 5-hydroxytryptamine rhythm at 17.00 h. can be correlated with the activity of 5-hydroxytryptophan decarboxylase, which is maximal at this time. The increase in concentrations of 5-hydroxytryptamine from 09.00 h. until 13.00 h. is explained by the increased activity of 5-hydroxytryptophan decarboxylase by the mechanism presented previously. The decrease from 13.00 h. to 01.00 h. can also be explained by decarboxylase activity as described before. However, this part of the rhythm is probably influenced to a significant extent by the reduced activity of 5-hydroxytryptamine-containing neurones, as reflected in the 5-hydroxyindole-3-acetic acid concentrations at these times. However, a second mechanism must be proposed for the increase of 5-hydroxytryptamine concentrations from 01.00 h. to 09.00h. since decarboxylase activity remains at a minimum during this period. It is significant perhaps that the concentrations of circulating and brain tryptophan are highest at 01.00 h. and it is therefore conceivable that the availability of the amino acid may in fact play a part in the initial increase in 5-hydroxytryptamine concentrations by methods which have not been detected in this work (mechanisms involving cyclic AMP may play a part). It is apparent however that tryptophan availability alone is insufficient to produce the twenty-four hour variation of 5-hydroxytryptamine in the rat brain.

Several factors which may participate in the control of the synthesis of 5-hydroxytryptamine in the CNS have not been investigated in this work. First, as already mentioned above, one of the most interesting and perhaps the most important omission is the role of cyclic AMP on the transport of tryptophan to 5-hydroxytryptamine-containing neurones. Some evidence is

available that concentrations of the nucleotide increase in situations where the synthesis of 5-hydroxytryptamine is stimulated (Cramer, Ng, and Chase 1972), and it has also been demonstrated that noradrenaline modulates cyclic AMP concentrations in the glial cells surrounding 5-hydroxytryptamine-containing neurones. The inference can therefore be drawn that cyclic AMP may contribute to changes in 5-hydroxytryptamine synthesis and to the interneuronal regulation of 5-hydroxytryptamine-containing neurones (Glowinski, Hamon and Hery 1973). Secondly, it has been reported that, in an experimental situation, the intraneuronal levels of 5-hydroxytryptamine appear to control the rate of synthesis of the amine in the spinal cord (Schubert 1973). However, this remains to be more fully elucidated.

Suggestions for further work

1) It would be of interest to correlate the interacting mechanisms of the twenty-four hour rhythms of catecholamines and 5-hydroxytryptamine in specific brain regions of the rat brain with reference to the availability of the amine precursors.
2) The effects of cyclic AMP concentrations on precursor availability have not been widely explored. It would be interesting to tackle this problem on the system presented in (1).
3) It is necessary to clarify the situation with regard to the activity of 5-hydroxytryptamine-containing neurones and 5-hydroxytryptamine concentrations, since the results reported here do not always agree with those reported by other workers.
4) Tryptophan-5-hydroxylase exists in both soluble and particulate forms (Knapp and Mandell 1972). It could prove of interest to determine whether these forms could have a cumulative effect on the production of 5-hydroxytryptamine at nerve endings, and whether there could be any variation with clock hour in the axonal rate of flow of the enzyme.

In more general terms it is evident that it is possible to use the twenty-four hour rhythms of noradrenaline and 5-hydroxytryptamine (which appear to be delicately balanced in opposition) to investigate interactions and the effects of drugs on these amines in the brain. These experiments may also have helped to elucidate some of the mechanisms involved in the control of 5-hydroxytryptamine synthesis.

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